Characterization of melatonin production and physiological functions in yeast

by

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Declaration

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Summary

Melatonin is a molecule that is found in all living organisms with numerous functions such as the regulation of circadian rhythms in animals and growth stimulation in plants. Studies suggest that melatonin acts as an antioxidant in all living organisms. Its biosynthetic pathways and biological functions are characterised in plants and animals but very little is known about these processes in microorganisms. Yeasts have been found to synthesise melatonin under various conditions such as fermentation, starvation and aerobic growth, but production patterns were found to be inconsistent. The purpose of this study was to investigate the physiological functions of melatonin in yeast in more detail by evaluating a large number of growth conditions to determine conditions that would elicit consistent melatonin production. The study used *Saccharomyces cerevisiae* as a model organism to study the impact of melatonin on cellular physiology and identify biosynthesis- and melatonin-responsive genes.

None of the conditions investigated in this study resulted in consistent melatonin production. When detected, concentrations of melatonin were very low (ng/10^7 cells) and varied greatly between biological repeats. In plants and animals, melatonin production oscillates in response to diurnal cycles; however, this oscillatory pattern was not observed in the current study. An analysis of the pathway intermediates suggest that multiple enzyme reactions may be involved in the synthesis of melatonin in yeast and that yeast appears not to possess a dedicated synthesis pathway. The absence of orthologs of the enzymes involved in the biosynthetic pathway in yeast supports this conclusion. The chaotic production pattern in yeast suggests that melatonin may be a product of non-specific enzymatic reactions or overflow metabolism.

This study took a different approach to evaluating the response of cultures to oxidative stress by conducting experiments in continuous culture conditions instead of batch culture. Over time, the number of differentially expressed genes decreased more rapidly together with yeast recovery from stress in melatonin treated cultures compared to melatonin untreated cultures. Transcriptomic analysis of *S. cerevisiae* treated with melatonin pre- and post-H_2O_2 induced oxidative stress suggests that it does not act through any specific stress-responsive pathway and its activity could not be linked to any specific genetic interaction or regulation. However, in the absence of stress, exogenous melatonin enhanced the expression of sulphate assimilation pathway genes. This pathway leads to the formation of methionine and cysteine which are involved in the production of glutathione, and the response therefore may prime cells for subsequent stress. When *S. cerevisiae* was stressed with various reactive oxygen species generating stressors, melatonin supplementation improved the survival of the cultures in a similar manner to other antioxidants, by increasing the expression of several genes that support the general antioxidant response.

Opsomming

Melatonien is ’n molekule wat in alle lewende organismes voorkom en wat verskeie funksies het, soos die regulering van sirkadiese ritmes in diere en groeistimulasie in plante. Studies stel voor dat melatonien as ’n antioksidant in alle lewende organismes optree. Die biosintetiese weë en biologiese funksies daarvan is in plante en diere gekarakteriseer, maar min is bekend oor hierdie prosesse in mikroörganismes. Gis is gevind om melatonien onder verskeie toestande te sintetiseer, soos tydens fermentasie, uithongering en aërobiese groei, maar produksiepatrone is gevind om inkonsekwent te wees. Die doel van hierdie studie was om die fisiologiese funksies van melatonien in gis in groter besonderhede te ondersoek deur ’n groot getal groeitoestande te evalueer om die toestande te bepaal wat konsekwente melatonienproduksie sal ontlok. Die studie het gebruik gemaak van *Saccharomyces cerevisiae* as ’n modelorganisme om die impak van melatonien op sellulêre fisiologie te bestudeer en om biosintese- en melatonien-responsiewe gene te identifiseer.

Geen van die toestande wat in hierdie studie ondersoek is, het gelei tot konsekwente melatonienproduksie nie. Wanneer dit bespeur is, was die konsentrasies melatonien baie laag (ng/10^7 selle) en het hulle grootliks tussen biologiese herhalings verskil. In plante en diere ossilleer melatonienproduksie in reaksie op dagsiklusse; hierdie ossillerende patroon is egter nie bespeur nie. ’n Analise van die tussenloperweë stel voor dat verskeie ensiemreaksies moontlik in die sintese van melatonien in gis betrokke is en dat die gis nie ’n toegewyde sinteseweg blyk te hê nie. Die afwesigheid van ortoloë van die ensieme wat in die biosintetiese weg in gis betrokke is, ondersteun hierdie gevolgtrekking. Die chaotiese produksiepatrone in gis stel voor dat melatonien moontlik ’n produk is van nie-spesifieke ensimatiese reaksies of oorvloei-metabolisme.

Hierdie studie het ’n verskillende benadering gevolg tot die evaluering van die reaksie van kulture op oksidatiewe stres deur eksperimente in deurlopende kultuurtoestande te onderneem in plaas daarvan om van lotkultuur gebruik te maak. Mettertyd het die aantal differensieel uitgedrukte gene vinniger verminder, tesame met gisherstel van die stres, in melatonienbehandelde kulture as in melatonien-onbehandelde kulture. Die transkriptomiese analise van *S. cerevisiae* wat vóór en ná H_2O_2-geïnduseerde oksidatiewe stres met melatonien behandel is, dui daarop dat dit nie deur enige spesifieke stresresponsiewe weg funksioneer nie en dat die aktiwiteit daarvan nie aan enige spesifieke genetiese interaksies of regulering gekoppel kan word nie. In die afwesigheid van stres, verhoog eksogene melatonien die uitdrukking van die gene van die sulfaat-assimilasieweg. Hierdie weg lei tot die vorming van metionien en sisteïen, wat betrokke is in die produksie van glutatioon, en die respons berei dus moontlik selle vir die gevolglike stres voor. Toe *S. cerevisiae* met verskillende reaktiewe suurstofspesies wat stressors genereer, gestres is, het melatonienaanvulling die gevolglike oorlewing van die kulture verbeter op ’n soortgelyke manier as ander antioksidante deur die uitdrukking van verskeie gene wat die algemene antioksidantrespons ondersteun, te verhoog.

This dissertation is dedicated to my family

I am who I am because of all your unwavering support.

Biographical sketch

Thato Motlhalamme was born and raised in Gaborone, Botswana. She matriculated from St Patricks College in Kimberley in 2003. She enrolled at the University of the Western Cape and obtained a degree in Complementary Medicine in 2010 followed by a MSc in Medical Biosciences in 2013. Thato then enrolled for a PhD-degree in Wine Biotechnology at Stellenbosch University in 2014.

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Preface

This dissertation is presented as a compilation of 6 chapters. Each chapter is introduced separately and is written according to the style of the journal Frontiers in Microbiology.

Chapter 1	**General Introduction and Project aims**
Chapter 2	**Literature review** Melatonin: current understanding of the biosynthetic pathways, biological functions and impact in macro- and micro- organisms
Chapter 3	**Research results** Characterization of melatonin production by yeast – an *in vitro* and *in silico* study
Chapter 4	**Research results** Melatonin modulates the transcriptional response of *S. cerevisiae* to hydrogen peroxide oxidative stress
Chapter 5	**Research results** Impact of melatonin supplementation on yeast stress responses
Chapter 6	**General discussion and conclusions**

Table of Contents

List of Abbreviations

3-IAA	3-indole acetic acid
5-HT	Serotonin
5-HTP	5-hydroxytryptophan
5-MT	5-methoxytryptamine
6-OHM	6-hydroxymelatonin
ADP	Adenosine diphosphate
AADC	Aromatic L-amino acid decarboxylase
AANAT	Arylalkylamine N-acetyltransferase
ASMT	N-acetylserotonin O-methyltransferase
BH4	Tetrahydrobiopterin
CBS	Centraal Bureau voor Skimmelcultures
cDNA	Complimentary DNA
COMT	Caffeic acid O-methyltransferase
C_T	Threshold cycle
$CuCl_2$	Cupric chloride
DEPC	Diethyl Pyrocarbonate
DEG	Differentially expressed genes
DHPR	6-pyruvovyl-tetrahydropterin synthase
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EPT1	EthanolaminePhosphoTransferase
ESI	Electrospray ionization
FDH1	Formate dehydrogenase 1
GC-MS	Gas chromatography mass spectrometry
GND1	6-phosphogluconate dehydrogenase 1
H_2O_2	Hydrogen peroxide
HIOMT	Hydroxyindole-O-methyltransferase
IEA	Tryptophol
IL	Interleukin
LC-MS	Liquid chromatography-mass spectrometry

LOD	Limit of detection
MEL	Melatonin
MM	Minimal medium
NaCl	Sodium chloride
NAT	Arylamine N-acetyltransferase
NB	Nutrient broth
OD	Optical density
OSR	Oxidative stress response
PCBD1	4a-hydroxytetrahydrobiopterin dehydratase
PCR	Polymerase chain reaction
PCT1	Choline-phosphate cytidylyltransferase
PCT1	Phosphate cytidylyltransferase
PPP	Pentose phosphate pathway
qPCR	Quantitative real-time PCR
RE	Relative expression
RNS	Reactive nitrogen species
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SGJ	Synthetic grape juice
SGM	Synthetic grape must
SNAT	Serotonin N-acetyltransferase
SPE3	Spermidine synthase
SPO14	Phospholipase D
T5H	Tryptamine 5-hydroxylase
TAL1	Transaldolase
TCA	Tricarboxylic acid
TDC	Tryptophan decarboxylase
TDH2	Glyceraldehyde 3-phosphate dehydrogenase
TDH3	Glyceraldehyde-3-phosphate dehydrogenase
TKL1	Transketolase
TPH	Tryptophan hydroxylase
TPI1	Triosephosphate isomerase

TPS1	Trehalose-6-phosphate synthase
TPS1	Trehalose-6-phosphate synthase
TRP	Tryptophan
TSL1	Trehalose synthase complex regulatory subunit
YNB	Yeast nitrogen base
YPD	Yeast Peptone Dextrose

Chapter 1

General Introduction and Project Aims

Chapter 1

General Introduction and Project Aims

1.1 Introduction

Melatonin (*N*-acetyle-5-methoxytryptamine) is a product of tryptophan metabolism that is present in numerous phylogenetically distant taxa. It is found in organisms such as bacteria, unicellular eukaryotes, macroalgae, fungi, plants, invertebrate and vertebrate species (Pandi-Perumal et al., 2006). Its presence in various clades supports an early evolutionary origin and possibly suggest a phylogenetically conserved molecular function for melatonin.

The biosynthetic pathway for melatonin has been widely studied in plants and animals, however a single phylogenetic origin is not easy to demonstrate. In all organisms studied thus far, biosynthetic pathways involve four enzymatic steps, but homologs of mammalian rate limiting enzymes in the classic biosynthetic pathway have not been found in all eukaryotes. In plants, six enzymes are known to be involved in biosynthetic pathway suggesting the existence of multiple pathways (Back et al., 2016). The biosynthetic pathway in microorganisms is unknown. Some studies suggest that the pathway in yeast might be similar to the animal biosynthetic pathway (Sprenger et al., 1999) whereas Tan et al (2016) hypothesised that the predominant biosynthesis pathway in yeast might be similar to that of plants. Data thus far show that melatonin can be synthesised by multiple enzymes of different specificities even within one species (Hardeland, 2008; Back et al., 2016).

Melatonin was initially believed to be an exclusive product of the pineal gland in vertebrates and was referred to as a neurohormone. Research has however shown it to be synthesized in many extrapineal cells, organs and tissues (Tan et al., 2007). Outside the animal kingdom, it has been quantified in more than 140 plant and plant preparations. Concentrations range from picograms to micrograms per gram of tissue (Gomez et al., 2012). Various yeast and bacterial species have been found to produce melatonin although production patterns were inconsistent and quantified concentrations were very low (Tilden et al., 1997; Sprenger et al., 1999; Vigentini et al., 2015; Jiao et al., 2016; Ma et al., 2017).

Melatonin modulates numerous physiological processes in animals which include sleep/wake cycle, reproductive physiology via receptor mediated pathways (Reiter et al.), antiapoptotic functions (Lei et al., 2004) as well as oncostatic activities against some form of cancers (Pandi-Perumal et al., 2006). Melatonin has been shown to improve the resistance of

plants to environmental stresses such as desiccation, temperature, ultraviolet light, heavy metal stress and pathogen infection (Kolár and Machácková, 2005; Pandi-Perumal et al., 2006 Hardeland, 2016). In addition, melatonin has growth promoting effects similar to the plant hormone indole-3-acetic acid (Hernández-Ruiz and Arnao, 2008). Melatonin's antioxidant claims are based on its ability to scavenge free radicals. It also indirectly up-regulates several antioxidative enzymes and down-regulates pro-oxidant enzymes (Pandi-Perumal et al., 2006).

1.2 Melatonin in yeast

Several research groups have investigated the ability of *Saccharomyces* and non-*Saccharomyces* yeast strains to produce melatonin in different growth conditions. The pioneering study by Sprenger et al (1999) found that following a starvation period in salt medium, *S. cerevisiae* formed high concentrations of melatonin in growth medium supplemented with tryptophan, serotonin or N-acetylserotonin. The data suggested that tryptophan concentrations as well as yeast growth phase played a role in the levels of melatonin produced by yeast. Other studies also suggested that melatonin is mainly produced in exponential growth phase (Rodriguez-Naranjo et al., 2012; Vigentini et al., 2015; Fernández-Cruz et al., 2017) and that higher concentrations of tryptophan in media resulted in higher concentration of extracellular melatonin (Rodriguez-Naranjo et al., 2012; Vigentini et al., 2015). In wine making, the concentration of melatonin increased during the fermentation indicating that yeasts might play a role in its biosynthesis. The role of melatonin in *Saccharomyces* is however still unclear but it appears to not be related to the circadian rhythms (Hardeland and Poeggeler, 2003). Recently melatonin was found to increase the expression of some antioxidant enzymes in H_2O_2 stressed cells (Bisquert et al., 2018; Vázquez et al., 2017). It also modulated cell fatty acid composition by increasing oleic and palmitoleic acids which lead to higher UFA/SFA ratios and subsequently a higher tolerance to H_2O_2 (Vázquez et al., 2018).

1.3 Scope and Objectives of the study

Research aims

Although studies have shown that yeast can produce melatonin, conditions that stimulate consistent production have not been identified. In addition to this, the biosynthetic pathway and physiological functions of melatonin in yeast are not fully understood. The aim of this research is to characterise the growth conditions that stimulate melatonin production by yeast, and to characterise its biosynthesis and physiological role. Elucidation of the regulation of

melatonin production would increase our understanding of the physiological role of melatonin in yeast and may allow targeted increases of its concentration in fermented beverages such as wine. To achieve these aims, the following research objectives were set:

1. Investigate the ability of different wine-related yeast species and strains to produce melatonin

    - Evaluation of laboratory media and environmental conditions
    - Assessment of the impact of the presence of intermediates in the biosynthetic pathway
    - Analysis of the biosynthesis of end products from tryptophan

2. Understand the physiological role of melatonin in yeast

    - Assessment of the response of yeast to environmental stress the in the presence and absence of melatonin
    - Transcriptomic RNAseq analysis of gene expression in response to melatonin supplementation and oxidative stress

3. Identify biosynthesis and melatonin responsive genes

    - Targeted expression analysis of selected genes using qPCR
    - Identification of genes that may encode for enzymes involved in melatonin production through *in-silico* analysis.

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Chapter 2

Melatonin: current understanding of the biosynthetic pathways, biological functions and impact in macro- and micro-organisms

Chapter 2

Melatonin: current understanding of the biosynthetic pathways, biological functions and impact in macro- and micro- organisms

2.1 Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine) is a product of tryptophan metabolism and occurs ubiquitously in nature. (Hardeland et al. 2011). This indoleamine was first identified as a neurohormone, and was originally thought to be synthesized exclusively in the mammalian pineal gland, but has since been detected in a unicellular alga (Pandi-Perumal et al., 2006), photosynthetic bacteria (Manchester et al., 1995; Tilden et al., 1997), fungi (Hardeland, 1999), protozoans and plants (Dubbels et al., 1995; Hattori et al., 1995).

Most research on melatonin in the past was performed on animal models or tissues and as a result the biosynthetic pathway, regulation of production and biological effects are well characterised in this kingdom. In animals, melatonin appears to play an important role in the regulation of many physiological events including sleep, circadian rhythms, body temperature, seasonal control of the reproduction system, retina physiology, appetite and immunological systems (Claustrat et al., 2005; Pandi-Perumal et al., 2006; Korkmaz et al., 2009; Tan et al., 2010). Melatonin also serves as an antioxidant molecule with free radical scavenging properties (García, 2014). In addition to this, there is evidence that melatonin possesses inhibitory effects on some form of cancers and shows beneficial effects on cardiovascular diseases (Hornedo-Ortega et al., 2016).

Since the discovery of this indoleamine in plants, various groups have investigated its biosynthetic pathway, regulation, localization and function in different plant species. It has been detected in roots, shoots, leaves, fruits and seeds in a variety of plant species (Dubbels et al., 1995; Hattori et al., 1995; Harumi and Matsushima, 2000) at concentrations that range from pg/g to mg/g of tissue (Posmyk and Janas, 2008). Studies have also elucidated the biosynthetic pathway which differs from the classic pathway described in animals with regard to enzymes involved as well as pathway intermediates (Back et al., 2016).

Investigations into the presence of melatonin in unicellular organisms and microorganisms have grown exponentially in the last two decades (Poeggeler and Hardeland, 1994; Manchester et al., 1995; Sprenger et al., 1999). Probiotic *Bifidobacterium* species (*breve, longum*), *Enterococcus* species (*faecalis* TH10), *Lactobacillus* species (*brevis, acidophilus, bulgaricus, casei, fermentum, helveticus, plantarum*) and *Streptococcus*

thermophiles have been used to produce melatonin on an industrial scale (Tan et al., 2012). Various species of yeast were found to synthesize melatonin during fermentation and are thought to be responsible for the presence of melatonin in various fermented food products (Rodriguez-Naranjo et al., 2011a; Vitalini et al., 2013; Fernandez-Cruz et al., 2019). The biological role and regulation of this indoleamine in microorganisms is not well understood. Recently studies have shown that it protects microorganisms against oxidative stress damage (Vázquez et al., 2017; Bisquert et al., 2018), but more work needs to be done to understand the regulation of the antioxidant activities observed.

This review will highlight occurrence of melatonin in all clades of living organisms as well as in a variety of food products. Several studies have challenged the view that the classic melatonin pathway is the predominant or sole pathway in organisms. This review will unpack our current understanding of the biosynthetic pathways that may be involved in the production of the indoleamine in animals, plants and microorganism with specific focus on yeast. We will also summarize the impact melatonin has on the biological functions in these clades.

2.2 Melatonin in various foods and beverages

The importance of melatonin to human health and the growing interest on the presence of this indoleamine in food (Hattori et al., 1995; Johns et al., 2013) has increased studies on melatonin by food scientists. These melatonin rich food sources could potentially be used as natural melatonin supplements and several studies found that consumption of melatonin rich foods resulted in increased levels found in human blood. Bonnefont-Rousselot & Collin (2010) found that the peak circulating levels of melatonin were 200 pg/mL and decrease to less than 10 pg/mL during the day. These concentrations are very low in comparison to levels found in some food products listed in Table 2.1. Another study investigating the impact of melatonin rich plant foods on human health reported that these foods increased the serum concentration of melatonin as well as the antioxidant capacity in healthy subjects (Sae-Teaw et al., 2013). As a result of this increase in research efforts, melatonin has been detected in many foods that form part of the human diet such as whole grains, fruits, vegetables, legumes, yogurt, bread (Kocadağli et al., 2014), wines from different types of fruit (Iriti et al., 2006; Mena et al., 2012; Wang et al., 2016), beers and animal products (Tan et al., 2014).

In plants, this indoleamine has been detected in roots, shoots, leaves, fruits and seeds in a variety of plant species (Dubbels et al., 1995; Hattori et al., 1995; Harumi and Matsushima, 2000) at concentrations that range from pg/g to mg/g of tissue (Manchester et al., 2000; Burkhardt et al., 2001; Posmyk and Janas, 2008; Sturtz et al., 2011). The concentrations of melatonin in plants seem to be influenced by the cultivars of plants, harvest time, degree of ripeness, and environmental conditions such as light intensity and agrochemical practices (Iriti

et al., 2006). Manchester et al. (2000) investigated the levels of melatonin in the seeds of edible plants and found that variations in the content of melatonin in the seeds. The lowest and highest concentrations were found in the milk-thistle seed and the white mustard respectively. Another study investigated the melatonin levels in different varietals of ripe strawberries and tomatoes across two seasons. In this study there was high inter-seasonal and varietal variability in melatonin concentrations reported in both fruits. Concentrations detected ranged from 1.38 – 11.26 ng/g and 4.11 – 114.52 ng/g in strawberries and tomatoes respectively (Sturtz et al., 2011).

Fermentation seems to play a role in the synthesis of melatonin in some food products. Rodriguez-Naranjo et al (2011a) found that the concentrations of melatonin increased during fermentation of grapes highlighting the role that yeasts play. Similar observations were found in mulberry wines where melatonin levels (12.5 ng/mL) were much higher than in unfermented fruits (1.41 ng/g). Red mulberry wines also had higher concentrations of melatonin in comparison to white wines which is in line with published data on grape wines (Wang et al., 2016). Other yeast fermented products such as beer and bread were found to have detectable amounts of melatonin (Table 2.1). Melatonin was also found in bacteria fermented products such as yogurt and naturally fermented black olives highlighting the role bacteria possibly play in the synthesis of melatonin in fermented products (Kocadağli et al., 2014). At an industrial scale, bacterial species such as *Bifidobacterium, Enterococcus*, *Lactobacillus* and *Streptococcus* are used to produce melatonin (Tan et al. 2012). Some of these bacterial species are used in the production of yogurt and cheese and could be the source of melatonin in these food products.

The presence of melatonin in meat products was described by Tan et al. (2014). In that study levels of melatonin detected were in the ng/g range which is comparable to those concentrations in other food stuffs such as cherries, coriander and corn (Manchester et al., 2000; Burkhardt et al., 2001; Kirakosyan et al., 2009). This antioxidant could improve the shelf life and taste of meats by preventing the oxidation of lipids and protein. In addition to this, consumption of melatonin rich meat could increase the blood circulation levels of melatonin in humans (Tan et al., 2014).

Table 2.1: Levels of melatonin reported in various food and beverage products.

Food sample	Melatonin concentrations	Reference
Fermented beverages		
Pomegranate wines	0.54 - 5.50 ng/mL	(Mena et al., 2012)
Hongguo (red) Mulberry	12.50-14.20 ng/mL	(Wang et al., 2016)
Baiyuwang (white) mulberry)	3.41-4.77 ng/mL	(Wang et al., 2016)
Beer	94.5 pg/mL	(Kocadağli et al., 2014)
Black olive (naturally fermented)	7.2 pg/g	(Kocadağli et al., 2014)
Bread crumb	341 pg/g	(Kocadağli et al., 2014)
Bread crust	138 pg/g	(Kocadağli et al., 2014)
Yogurt	126 pg/g	(Kocadağli et al., 2014)
Fruits and vegetables		
Strawberry	1.38 – 11.26 ng/g	(Sturtz et al., 2011)
Tomato	4.11 – 114.52 ng/g	(Sturtz et al., 2011)
Montmorency cherries	13.46 ng/g 12.3 ng/g	(Burkhardt et al., 2001) (Kirakosyan et al., 2009)
Balaton cherries	2.06 ng/g 2.9 ng/g	(Burkhardt et al., 2001) (Kirakosyan et al., 2009)
Milk thistle seed	2 ng/g	(Manchester et al., 2000)
Coriander	7 ng/g	(Manchester et al., 2000)
Flax seed	12 ng/g	(Manchester et al., 2000)
Alfalfa seed	16 ng/g	(Manchester et al., 2000)
Fennel seed	28 ng/g	(Manchester et al., 2000)
Almond	39 ng/g	(Manchester et al., 2000)
Fenugreek seed	43 ng/g	(Manchester et al., 2000)
Black mustard seed	129 ng/g	(Manchester et al., 2000)
White mustard seed	189 ng/g	(Manchester et al., 2000)
Extra virgin olive oil	71-119 pg/mL	(de la Puerta et al., 2007)
Whole yellow corn	1.3 ng/g	(Tan et al., 2014)
Corn germ meal	1.0 ng/g	(Tan et al., 2014)
Green coffee	39.0 pg/mL	(Kocadağli et al., 2014)
Barley	0.58 ng/g	(Hernández-Ruiz and Arnao, 2008)
Animal products		
Salmon	3.7 ng/g	(Tan et al., 2014)

Whole chicken	2.3 ng/g	(Tan et al., 2014)
Lamb	1.6 ng/g	(Tan et al., 2014)
Mixture of chicken liver and heart	1.1 ng/g	(Tan et al., 2014)
Colostrum powder	0.6 ng/g	(Tan et al., 2014)
Beef	2.1 ng/g	(Tan et al., 2014)
Pork	2.5 ng/g	(Tan et al., 2014)
Dried egg solids	6.1 ng/g	(Tan et al., 2014)

2.2.1 Melatonin in grape cultivars and wine

Iriti et al. (2006) first reported the presence of melatonin in several grape varieties (Table 2.2). Many other research groups have confirmed the presence of this indoleamine in grapes but there are variations in the concentrations reported. These variations seem to be dependent on the cultivar, degree of ripeness and harvest time. Melatonin levels reported in wine vary greatly even in the same cultivar (Table 2.2). Studies on Cabernet sauvignon and Merlot grown in Spain and Italy found different concentrations of melatonin in these wines. Concentrations ranged from 0.32-74 ng/mL for Cabernet sauvignon and 4.4 - 245.46 ng/mL for Merlot. Rodriguez-Naranjo et al. (2011a, 2011b, 2013) reported three different concentrations of melatonin in Tempranillo wines from the 2007 and 2010 vintages (5.5 – 306.86 ng/mL). It should be mentioned that the differences in melatonin levels reported could be due to the analytical method used to quantify this indoleamine. Indeed, early studies on the indoleamine used immunoassays as detection methods but were limited in the sensitivity and specificity of the antibodies used resulting in overestimation of melatonin levels or possibility of false positive findings (Garcia-Parrilla et al., 2009). Chromatographic techniques such as gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS) were found to be more sensitive and specific in detecting melatonin. Different studies coupled liquid chromatography with tandem mass spectrometry or fluorescence detection to improve the accuracy of melatonin in food matrices. In the study by Rodriguez-Naranjo et al. (2011a) wine samples were concentrated by dehydration and reconstituted in methanol:water solution before LC-ESI (electrospray ionization)-MS/MS whereas the Rodriguez-Naranjo et al. (2013) study used solid-phase extraction to concentrate samples. The melatonin concentrations in Tempranillo wines reported in the former study were approximately fifty times higher than the latter. Climatic changes in each season may have also impacted the concentration of the precursors resulting in the different melatonin concentrations in Tempranillo wines from the same vineyard. The data published thus far does not show a terroir specific impact on melatonin concentration in grapes and wine. Melatonin levels in some red

wines have been found to be higher than in some white varietals. However, white varietals such as Prieto Picudo (49.0 ng/mL) and Palomino fino (390.82 ng/mL) had higher levels of melatonin in comparison to other red varietals (Table 2.2). Vitalini et al. (2013) suggested that these variations maybe due to winemaking practices that facilitate increased extraction of melatonin from the berry skin and seeds. The authors also explained that the quicker maceration time, filtration and pasteurisation of grape juice may result in the lower levels of melatonin.

Rodriguez-Naranjo et al. (2011a) found that melatonin was detected in grape must only after inoculation with yeast and increased during fermentation. Tan et al. (2012) hypothesised that *S. cerevisiae* synthesise melatonin as a stress response to the alcohol formed during alcoholic fermentation. This hypothesis was partially supported by Rodriguez-Naranjo et al (2011a) where melatonin was found on the fifth day of fermentation and the alcohol concentration at that point was approximately 11.1%. However, the relationship between alcohol content in wines and melatonin production is not consistent as the indoleamine concentrations varied considerably during fermentation in other studies (Fernández-Cruz et al., 2017, 2018). The role of bacteria in melatonin production in wine has not been extensively explored even though Rodriguez-Naranjo et al. (2013) found that melatonin content increases from 5.6 to 18 µg/L in Tintilla de Rota after malolactic fermentation. This was the only varietal that had an increase in melatonin after malolactic fermentation in this study, but further studies are needed to elucidate the melatonin production by bacteria during fermentation.

Table 2.2: Levels of melatonin reported in various grape cultivars and wine products

Cultivar	Melatonin concentrations		Reference
	Grape	Wine	
Red cultivars			
Cabernet Franc	0.005 ng/g		(Iriti et al., 2006)
Marzemino	0.031 ng/g		(Iriti et al., 2006)
Merlot	0.264 ng/g 150 µg/g	245.46 ng/mL 4.4-5.5 ng/mL	(Iriti et al., 2006) (Murch et al., 2010)(Rodriguez-Naranjo et al., 2011a)(Vitalini et al., 2011)
Sangiovese	0.332 ng/g	0.4 ng/mL	(Iriti et al., 2006) (Mercolini et al., 2008, 2012)
Cabernet Sauvignon	0.422 ng/g	0.32 ng/mL 14.2 ng/mL 74.13 ng/mL	(Iriti et al., 2006; Rodriguez-Naranjo

			et al., 2011b, 2011a)
Barbera	0.633 ng/g		(Iriti et al., 2006)
Croatina	0.870 ng/g		(Iriti et al., 2006)
Nebbiolo	0.965 ng/g		(Iriti et al., 2006)
Tempranillo		130 ng/mL 77.72 ng/mL 306.86 ng/mL	(Rodriguez-Naranjo et al., 2011a, 2011b)
Albana		0.6 ng/mL	(Mercolini et al., 2008, 2012)
Tintilla de rota (Graciano)		322.68 ng/mL	(Rodriguez-Naranjo et al., 2011a)
Gropello		5.2-8.1 ng/mL	(Vitalini et al. 2011)
Malbec		0.24 ng/mL	(Stege et al., 2010)
Petit Verdot		5.1 ng/mL	(Rodriguez-Naranjo et al., 2011b)
Syrah (Shiraz)		86.5 ng/mL 423.03 ng/mL	(Rodriguez-Naranjo et al., 2011a, 2011b)
White varietals			
Palomino fino		390.82 ng/mL	(Rodriguez-Naranjo et al., 2011a)
Trebbiano		0.4 ng/mL	(Mercolini et al., 2008, 2012)
Prieto Picudo		49.0 ng/mL	(Rodriguez-Naranjo et al., 2011b)

2.3 Melatonin and isomers structure and metabolites

The chemical structure of melatonin was first described by Lerner et al. (1959). It is a low molecular weight, amphiphilic molecule (Shida et al., 1994; Ceraulo et al., 1999) with the ability to cross cell membranes and can therefore be found in any aqueous compartment such as the cytosol, nucleus and mitochondrion (Acuña-Castroviejo et al., 2001, 2003). Melatonin has an indole ring with two side chains, which are a methoxy group at position 5 and N-acetylaminoethyl group at position 3 (Tan et al., 2012) (Fig 2.1). The carbonyl group in the N-acetylaminoethyl side chain plays a key role in the ability of melatonin to scavenge reactive species (e.g. peroxides, superoxide and nitric oxide) whereas the nitrogen is necessary for melatonin to form a new five membered ring after interacting with reactive species resulting in the formation of cyclic 3-hydroxymelatonin. The methoxy group seems to prevent melatonin

from exhibiting prooxidative activity (Tan et al., 2002). 3-Hydroxymelatonin scavenges radical species resulting in the formation of N1-acetyl-N2-formyl-5-methoxyknuramine which also scavenges radical species and is metabolised to N^1-acetyl- 5-methoxyknuramine (Tan et al., 2012; Reiter et al., 2014). The ability of melatonin and its metabolites to neutralize numerous toxic oxygen derivatives is referred as its cascade which distinguishes it from other classic antioxidants. Through this cascade reaction, one melatonin molecule can scavenge up to ten reactive oxygen species (ROS) molecules whereas other antioxidants scavenge ROS molecules on a 1:1 ratio (Tan et al., 2015).

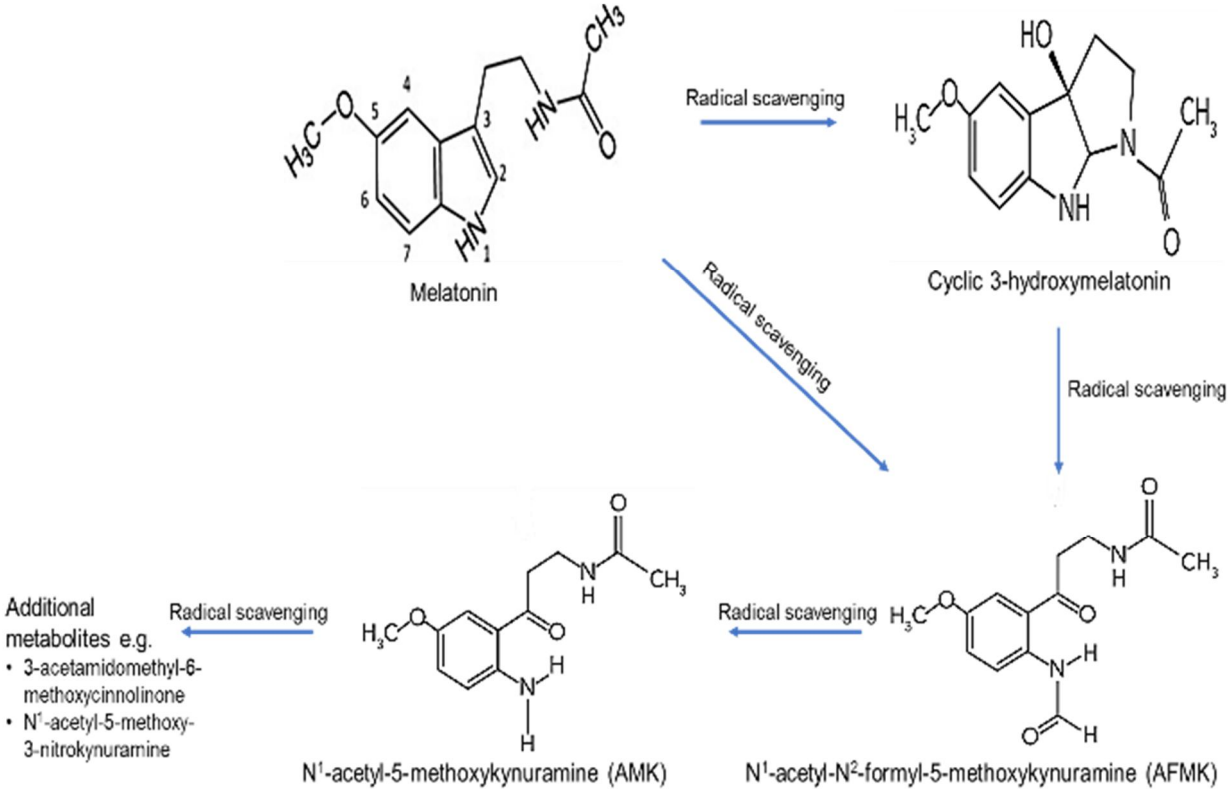

Figure 2.1: Chemical structures of melatonin with numbers identifying positions of the indole ring as well as metabolites formed when melatonin scavenges toxic radicals. Adapted from Tan et al (2012) and Reiter et al (2014).

The evolving research into melatonin has recently led to the discovery of melatonin isomers in wine and other fermented food products (Rodriguez-Naranjo et al., 2011; Tan et al., 2012; Vitalini et al., 2013; Vigentini et al., 2015). The position of the two side chains in the melatonin structure (Fig 2.1) determine the isomer formed. The methoxy group at position 5 and the N-acetylaminoethyl group at position 3 could hypothetically be relocated to any of the seven positions on the indole nucleus to form an isomer (Fig 2.2). Tan et al. (2012) calculated that 42 potential melatonin isomers could exist based on combinations of these side chains at

7 different positions on the indole ring. Levels of melatonin isomers in wine reported in literature are significantly higher than melatonin. However, current data also show that melatonin does not have a direct correlation to the formation of melatonin isomers. These findings suggest that isomers are not modified from melatonin but rather generated from other pathways (Tan et al. 2012). One of the most abundant melatonin isomers was found to not be an isomer but rather a tryptophan ethylester (Gardana et al., 2014).

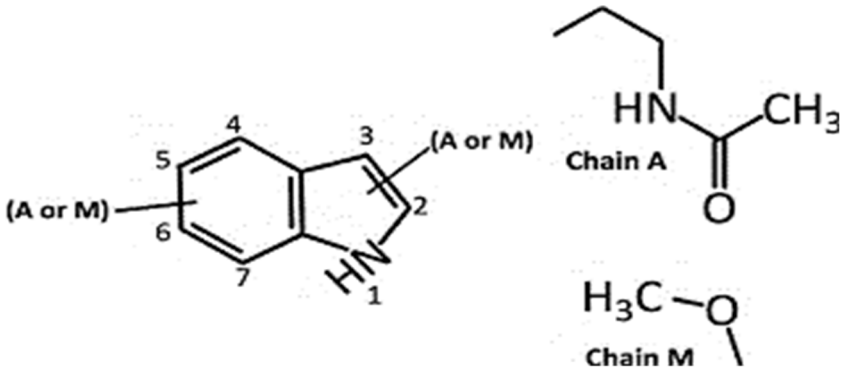

Figure 2.2: Chemical structure of the proposed melatonin isomers. The position of the two side chains on the indole ring determine the isomer formed (Tan et al. 2012).

2.4 Biosynthesis and regulation of melatonin

The discovery of melatonin in organisms outside the animal kingdom (Pöggeler et al., 1991) led to expansion of melatonin research in other clades. The biosynthetic pathways of melatonin have been extensively studied in animals and more recently in plants (Axelrod & Weissbach 1960; Back et al. 2016) and they have been shown to differ. Melatonin production by animals is limited by the low concentrations of the precursor amino acid tryptophan (Tan et al., 2016). In plants, the production of melatonin is not limited by the uptake of this amino acid. Plants that possess the shikimic pathway are able to synthesise tryptophan using the substrates D-erythrose-phosphate and phosphoenolpyruvate and phototrophs primarily use carbon dioxide (Bochkov et al., 2012). For this reason the levels of melatonin produced by plants are much higher compared to vertebrates (Fuhrberg et al., 1997; Sprenger et al., 1999; Chen et al., 2003). Although the biosynthesis of melatonin in microorganisms is not well understood, the pathways described in literature thus far for all organisms point to four enzymatic steps between the precursor amino acid tryptophan and melatonin (Back et al., 2016; Tan et al., 2016).

The nomenclature of some of the melatonin biosynthetic enzymes have been incorrectly used interchangeably in some studies. Tan et al. (2016) clarified the nomenclature of these enzymes based on the substrate specificity. Arylalkylamine N-acetyltransferase (AANAT), arylamine N-acetyltransferase (serotonin N-acetyltransferase (SNAT)) and arylamine N-acetyltransferase (NAT) catalyse the acetylation of indoleamines in a substrate and concentration dependent manner. SNAT however is selective for indole-ethylamines whereas NAT is not selective for indoles. Comparisons of the plant and cyanobacteria SNAT to AANAT amino acid sequences showed that these enzymes were not homologous and thus classified as orthologs. N-acetylserotonin O-methyltransferase (ASMT) is also known as hydroxyindole-O-methyltransferase (HIOMT). The O-methylating class of enzymes were found to be even more diverse. Plants have three sub-forms of ASMT and a species dependent activity of caffeic acid O-methyltransferase (COMT).

2.4.1 Biosynthetic pathway in animals

In vertebrates, the pathway for melatonin synthesis has been fully characterised (Hardeland et al., 2011). Tryptophan serves as the initial precursor in the pathway used to produce melatonin and the enzymes involved in this pathway include tryptophan hydroxylase (TPH), aromatic L-amino acid decarboxylase (AADC), ASMT/HIOMT, and AANAT/SNAT. TPH enzymes are part of the aromatic amino acid hydroxylases family and require tetrahydrobiopterin (BH4) as a co-substrate (Fitzpatrick, 1999). Tetrahydrobiopterin is produced by a separate pathway from GTP which involves from GTP cyclohydrolase I, 6-pyruvoyl-tetrahydropterin synthase and sepiapterin reductase (Thöny et al., 2000). In the classic pathway, TPH hydroxylates tryptophan to 5-hydroxytryptophan (5-HTP) and AADC converts 5-HTP to serotonin (5-hydroxytryptamine/5-HT). The enzyme activity of ASMT/HIOMT reaction with N-acetylserotonin is 14 greater in comparison to its reaction with serotonin (Axelrod and Weissbach, 1961), making N-acetylserotonin the preferred substrate for ASMT/HIOMT. Based on these findings, serotonin is first acetylated to *N*-acetylserotonin by AANAT/SNAT which is then methylated to melatonin by ASMT /HIOMT (Fig 2.3). It is believed that AANAT/SNAT is the rate-limiting enzyme for melatonin synthesis in vertebrates; however ASMT/HIOMT may also play the same role in the regulation of the maximum nocturnal melatonin concentration (Liu and Borjigin, 2005). In most cases there is a good correlation between the AANAT/SNAT activity and the environmental photoperiodic changes to which organisms are exposed to. In the pineal gland of vertebrates, the activity of AANAT/SNAT is light sensitive (inhibited by light and enhanced by darkness). This process is controlled by circadian oscillator as well as a phototic shutoff mechanism (Hardeland, 2008). Recently, it has been suggested that the classic pathway as described above is not the only

biosynthetic pathway in animals and may also not be the dominant pathway especially in extra pineal sites (Tan et al., 2016). This hypothesised pathway from serotonin via 5-methoxytryptamine (5-MT) to melatonin is based on data that demonstrated increased levels of melatonin after 5-MT administration in animals. In addition to this, comparisons of substrate specificities of AANAT in invertebrates revealed a preference for 5-MT rather than serotonin (Ichihara et al., 2001). Although more detailed studies are needed to confirm this hypothesis, this alternative pathway could contribute to melatonin levels produced in pineal and extra pineal sites.

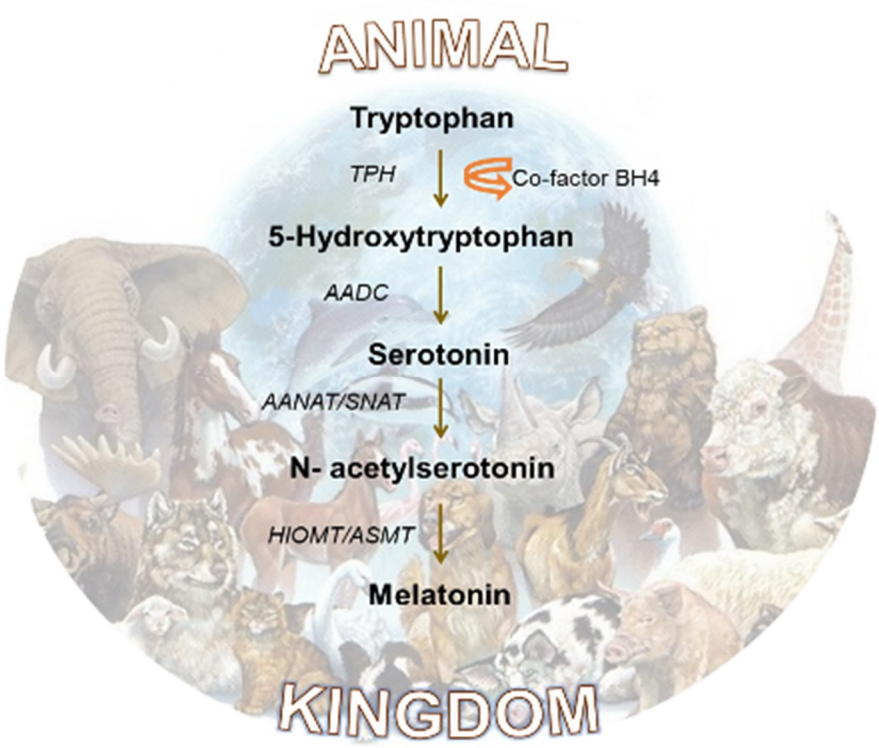

Figure 2.3: The classic melatonin biosynthetic pathway in animals. TPH, tryptophan hydroxylase; BH4, tetrahydrobiopterin; AADC, aromatic L-amino acid decarboxylase; AANAT, arylalkylamine N-acetyltransferase; SNAT, serotonin N-acetyltransferase; ASMT, N-acetylserotonin methyltransferase; HIOMT, hydroxyindole-O-methyltransferase (Axelrod and Weissbach, 1960; Weissbach et al., 1960)

2.4.2 Biosynthetic pathway in plants

The biosynthetic pathway of melatonin in plants, like the animal pathway, involves four enzymatic steps from tryptophan to melatonin. In plants however, six enzymes are known to be involved in this process suggesting the existence of multiple pathways (Back et al., 2016). These enzymes are tryptophan decarboxylase (TDC), TPH, tryptamine 5- hydroxylase (T5H), SNAT, ASMT, and COMT. Several studies have suggested that plants may have TPH-like genes although the actual genes have yet to be identified in the plant genome (Back et al., 2016). 5-HTP was found in high levels in the seeds of *Griffonia simplicifolia* (Lemaire and

Adosraku, 2002) and the BH4 dependent amino acid hydroxylase activity similar to TPH was found in the soluble fraction of rice root extract (Kang et al., 2007). The first two steps in the plant pathway differ from the animal pathway in that tryptophan is first decarboxylated to tryptamine by TDC in the cytoplasm and tryptamine is hydroxylated by T5H to serotonin in the endoplasmic reticulum (Fig 2.4). The last two steps in the biosynthesis of melatonin involve three enzymes SNAT, ASMT and COMT. Experimental evidence suggests that SNAT has substrate affinity for serotonin and 5-methoxytryptamine whereas ASMT and COMT has substrate affinity for serotonin and N-acetylserotonin. Serotonin could be transformed to 5-methoxytryptamine in the cytoplasm by the methytransferase ASMT/COMT and then transformed to melatonin by the *N*-acetyltransferase SNAT (Fig 2.4A). In an alternative pathway SNAT converts serotonin to *N*-acetylserotonin in the chloroplast which is then converted to melatonin by ASMT/COMT in the cytoplasm (Fig 2.4B) (Back et al., 2016).

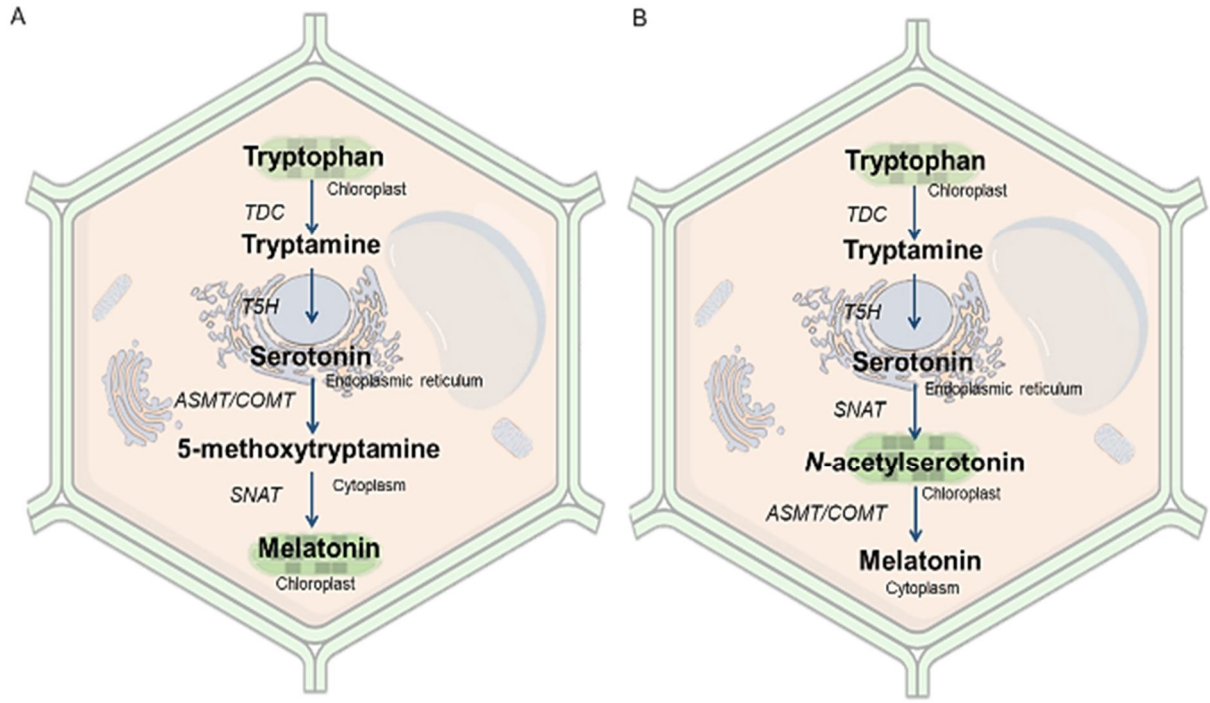

Figure 2.4: Multiple pathways of melatonin biosynthesis in plants based on the position of the intermediates and enzymes. TDC, tryptophan decarboxylase; T5H, tryptamine 5-hydroxylase; SNAT, serotonin N-acetyltransferase; ASMT, N-acetylserotonin methyltransferase; COMT, caffeic acid O-methyltransferase. Adapted from Back et al., 2016.

In the pathways with TPH activity, tryptophan is hydroxylated to 5-hydroxytryptophan in the cytoplasm by TPH which is then decarboxylated by TDC to serotonin in the cytoplasm (Fig 2.5 A and B). Thereafter serotonin could be converted as described above to either 5-methoxytryptamine or *N*-acetylserotonin which are then converted to melatonin. In all the possible biosynthetic pathways described in literature, serotonin seems to be an important intermediate in plants.

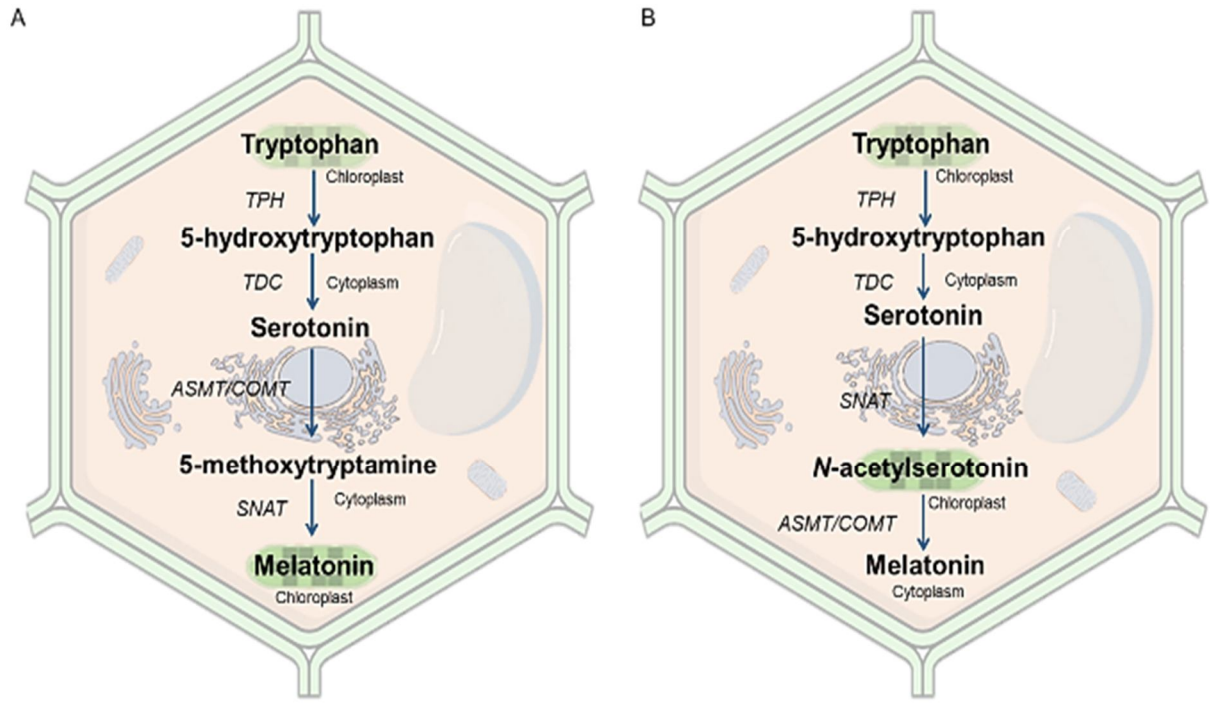

Figure 2.5: Melatonin biosynthetic pathways in plants that involve TPH. . TPH, tryptophan hydroxylase; TDC, tryptophan decarboxylase; SNAT, serotonin N-acetyltransferase; ASMT, N-acetylserotonin methyltransferase; COMT, caffeic acid O-methyltransferase. Adapted from Back et al., 2016.

2.4.3 Putative pathway in microorganisms

The biosynthetic pathway of melatonin in microorganisms remains to be identified with certainty. Sprenger et al. (1999) suggested that melatonin was possibly synthesized by *S. cerevisiae* via the pathway similar to that found in animals. This hypothesis was based on the initial studies when the yeast was incubated in the presence of tryptophan and the intermediates, serotonin and *N*-acetylserotonin resulted in melatonin accumulation (Figure 2.6). Their results also indicated that an alternative route from serotonin to melatonin may occur via 5-methoxytryptamine similar to that found in plants (Tan et al. 2016). To date, no reports have found or characterized the enzymes responsible for melatonin synthesis and genes encoding the enzymes in *S. cerevisiae* or any other yeast with one exception. Ganguly et al. (2001) cloned and characterized the *S. cerevisiae* homolog of AANAT and kinetic studies found that this enzyme had greater catalytic efficiency with 5-methoxytryptamine than with serotonin, suggesting that it may be the final enzyme in the biosynthesis pathway of melatonin. This result strengthened the hypothesis that the predominant biosynthesis pathway in yeast might be similar to that of plants (Tan et al. 2016). Furthermore, the high levels of 5-methoxytryptamine accumulation observed in the study of Sprenger et al. (1999) adds additional support.

Muñiz-Calvo et al. (2019) used a different strategy to unveil the putative melatonin biosynthetic pathway in *S. cerevisiae* by pulsing pathway intermediates in cells at different growth stages and analysing intracellular and extracellular samples for the presence of indolic compounds. The data generated suggested that the first step in the pathway differs from the classic pathway in that tryptophan is decarboxylated to tryptamine instead of 5-hydroxytryptophan. Tryptamine was then hydroxylated to serotonin which was then converted to N-acetylserotonin and 5-methoxytryptamine. But the presence of a metabolite in intracellular samples did not always correspond with its presence in extracellular samples and vice versa. For example, melatonin was detected at high concentrations in the extracellular samples and low levels in intracellular samples when 5-methoxytryptamine was pulsed into synthetic defined medium. The impact of metabolite pulsing on production of other metabolites was also affected by the composition of the medium and this pulsing did not result in the production of all downstream metabolites. The data generated thus far suggests that yeast may utilise more than one pathway to synthesis melatonin.

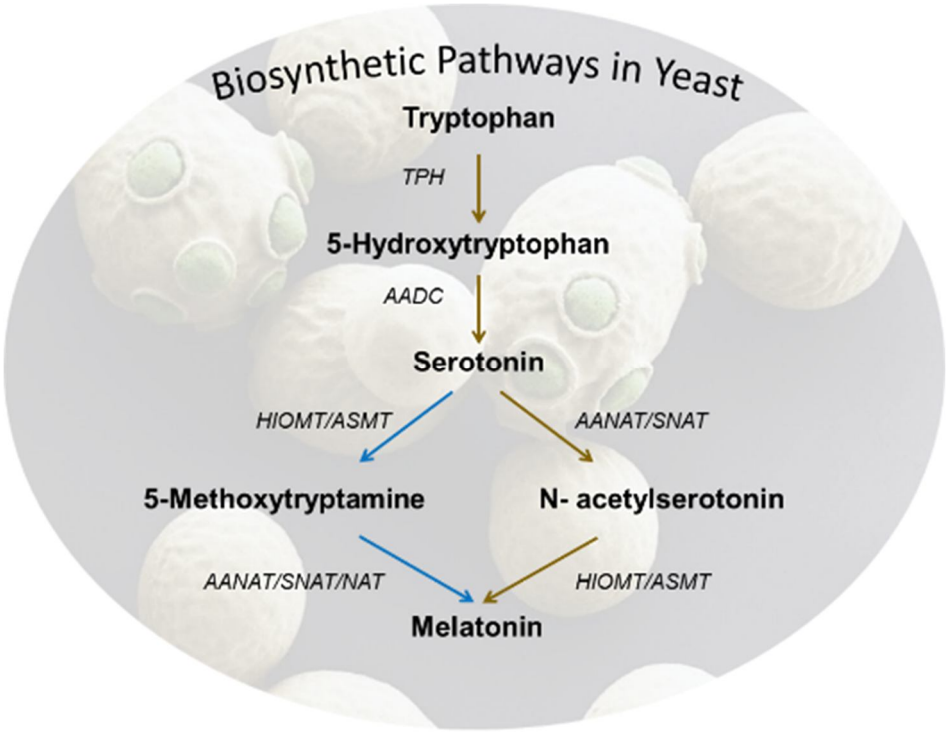

Figure 2.6: Hypothetical melatonin pathway in yeast. TPH, tryptophan hydroxylase; AADC, aromatic L-amino acid decarboxylase; AANAT, arylalkylamine N-acetyltransferase; SNAT, serotonin N-acetyltransferase; ASMT, N-acetylserotonin methyltransferase; HIOMT, hydroxyindole-O-methyltransferase. Adapted from Sprenger et al. 1999 and Tan et al. 2016.

2.5 Functions of melatonin

Since the discovery of melatonin, numerous studies have investigated the biological functions of this molecule in various organisms and found that this molecule has a broad spectrum of physiological functions but a common function in all organisms is free radical scavenging and antioxidant activities (Pandi-Perumal et al., 2006). *In vivo* and *in vitro* studies have shown that the antioxidant capacity of melatonin is higher than other classic antioxidants such as vitamin C, vitamin E, glutathione and NADH (Gitto et al., 2001; Tan et al., 2003b). The main reason for this is the cascade reaction of melatonin with ROS and reactive nitrogen species (RNS) where the metabolites of melatonin retain free radical scavenging abilities. In addition to this, melatonin synthesis is induced by moderate oxidative stress and exogenous application of this molecule increases the messenger RNA and protein levels of other antioxidant enzymes (Mayo et al., 2002; Tan et al., 2015). These unique features improve the efficiency of melatonin in protecting organisms from stressful conditions.

Melatonin's characteristic diurnal changes made it an ideal signalling molecule during evolution to transduce the environmental photoperiodic information. In primitive photosynthetic bacteria, the diurnal changes in melatonin levels occurred as a result of increased utilization during photosynthesis which generates large quantities of ROS (Manchester et al., 1995; Tilden et al., 1997). This diurnal rhythm of melatonin in bacteria likely evolved into the bio-clock in higher organisms (Tan et al., 2010, 2015) which regulates sleep cycles, reproductive cycles and hibernatory behaviours in animals (Reiter, 1993; Tan et al., 2015). Disruptions to the circadian rhythm of melatonin is associated with health problems such as cancer, heart disease and hypertension as well as neurodegenerative disorders. The circadian rhythm of melatonin in plants does not follow the same pattern observed in animals with peak concentrations at night and low concentrations during the day. In plants, melatonin production is stimulated by light exposure and increased light intensity results in increased melatonin production (Murch et al., 2000; Afreen et al., 2006; Tan et al., 2007). The circadian rhythm of melatonin in all organisms relates to its original primary role as an antioxidant which promotes stress resistance, repair and survival of organisms.

2.5.1 Animals: circadian rhythm and stress

As organisms evolved from unicellular to multicellular, internal cells and organs of larger species lost the ability to directly respond to circadian photoperiodic changes. It is hypothesised that these organisms adopted melatonin's diurnal cycle and this indoleamine became a photoperiodic signalling molecule (Tan et al., 2010). Melatonin circadian rhythm in vertebrates is regulated by a restricted bandwidth of visible light (wavelengths from 460-480 nm/blue light) (Reiter et al., 2014) and is mediated by sophisticated chain reactions that involve

retinal photo-reception, neuroelectro-chemical transformation, neural transmission and transduction of a neural message into a chemical output (Reppert, 1997). This fraction of daylight inhibits melatonin synthesis in the pineal gland during the day resulting in maximal production at night. However, melatonin produced by the pineal gland is only a small part of the total melatonin produced in multicellular organisms. Various organs and tissues were found to synthesise melatonin at higher concentrations to those produced by the pineal gland (Huether, 1993; Tan et al., 2010). Nevertheless, unlike the pineal gland, extra pineal melatonin is not released into the bloodstream but rather used in the tissues in which it is produced and functions as a tissue factor, autacoid and paracoid (Tan et al., 2003a).

As animals evolved, they adapted to survive harsh environmental conditions such as temperature fluctuations, famine and infections. These mechanisms included development of an immune system, regulation of reproductive functions and thermogenic processes in response to environmental conditions (Lochmiller and Deerenberg, 2000). The stress associated with low winter temperatures, scarce food sources and seasonal infections have been shown to have immunosuppressive effects (Demas and Nelson, 1998) and in order to survive these stressors, the activity immune system is up-regulated. Animals adopted the photoperiodic properties of melatonin as a seasonal indicator and several studies have demonstrated the relationship between immunomodulation and melatonin synthesis (Miller et al., 2006; Reiter et al., 2005). The indoleamine has been shown to be involved in the regulation of both cellular and humoral immunity. It stimulates the production of natural killer cells, monocytes and leukocytes, cytokines such as interleukin (IL)-2, IL-6, IL-12 and interferon-gamma and alters the balance of T helper (Th)-1 and Th-2 cells (Pandi-Perumal et al., 2006; Srinivasan et al., 2008). Organisms elicit an inflammatory response to protect against bacterial, viral and parasitic infection but an overreaction if this response can result in serious tissue and organ damage. Melatonin modulates this inflammatory response by down-regulating the expression of the pro-oxidant enzymes cyclooxygenase-2 and inducible nitric oxide synthase and inhibits the activities of myeloperoxidase and eosinophilic peroxidase.

As an antioxidant, melatonin preserves cell viability by protecting lipids, proteins and DNA from ROS induced damage (Fernández-Mar et al., 2012; García, 2014). The chronobiotic, antioxidant, anti-inflammatory and immunomodulating activities exhibited by melatonin may play an important role in protecting cells and organs against several pathophysiological conditions such as cancer, hypertension, sepsis, Alzheimer's and Parkinson's disease.

2.5.2 Plants: a role in abiotic stress protection, growth and maturation

Melatonin has multiple functions in plants (Fig 2.7) which include root development, seed germination, fruit ripening, senescence and yield. This molecule also plays a key role in a plant's response to abiotic and biotic stress by activating different defence mechanisms (Hardeland, 2016). Abiotic stresses such as heat, cold, drought, salt and chemical stressors generate ROS in cells which can damage cellular membranes, DNA, protein and enzyme activity (Yu et al., 2018). Exposure to these stresses enhanced the production of melatonin in various plants (Tan et al., 2015; Shi et al., 2016) and the increase in the number of studies on melatonin in plants has deepened our understanding on the molecular mechanisms of melatonin mediated stress responses in plants.

Some studies found that under stress conditions, the gene expression and enzyme activity of some of the melatonin biosynthesis pathway intermediates were modulated. Byeon et al. (2014, 2015) found that cadmium and heat stress increased the expression of TDC, T5H, ASMT and SNAT in rice which increased melatonin production. Similar observations were found in two species of apple where drought conditions up-regulated TDC, T5H, SNAT, and HIOMT genes (Li et al., 2015). Once synthesised, melatonin can directly scavenge the ROS generated by these stresses and in the process, it is metabolized to a number of products (2-hydroxymelatonin, cyclic-3-hydroxymelatonin and *N*1-acetyl-*N*2-formyl-5-meth-oxyknuramine) which also have ROS scavenging abilities (Hardeland, 2016). In addition to this, melatonin has been shown to activate ROS scavenging enzyme systems such as superoxide dismutase, ascorbate peroxidase, catalase and glutathione peroxidase in response to salt and phytotoxic stress in *Arabidopsis*; vanadium stress in watermelon; drought stress in apples (Li et al., 2012; Nawaz et al., 2018). Melatonin also activated the production of antioxidants ascorbic acid, glutathione in drought stressed wheat seedlings and cold stressed tomato plants. In addition to scavenging ROS and activating antioxidants, melatonin improved heavy metal stress resistance of tomato through sequestration of the metal (cadmium) by phytochelatins. Recently melatonin was found to mediate the induction of transcription factors involved in a stress dependent manner. These melatonin mediated transcription factors include oxidant induced zinc finger transcription factors, heat shock factors and C-Repeat-Binding factor/Drought response element binding 1 factors (Hardeland, 2016).

One of the early roles proposed for melatonin in plants was its activity as a growth regulator. Exogenous melatonin induced active growth of hypocotyls at micromolar concentrations in a similar manner to the plant hormone indole acetic acid and the highest concentration of melatonin was found in the apical zone which was the most actively growing zone (Hernández-Ruiz et al., 2004). Application of exogenous melatonin increased seed

germination rate and root growth of water stressed cucumber plants and minimizes the impact of the water stress (Zhang et al., 2013). Apple seedlings pre-treated with melatonin and subjected to salt stress had better shoot height, leaf number, chlorophyll content, and electrolyte leakage in comparison to untreated plants (Li et al., 2012). This indoleamine was also shown to have an impact on photosynthetic processes. It improved the efficiency of photosystem II, maintained a higher capacity for CO_2 assimilation and stomatal conductance as well as reducing chlorophyll degradation in drought stressed apple trees and water stressed cucumber seedlings (Arnao and Hernández-Ruiz, 2015). By enhancing the ROS scavenging enzyme activities which eliminated excess hydrogen peroxide (H_2O_2) and maintaining ascorbic acid and glutathione content, melatonin treatment delayed dark-induced senescence in apple leaves.

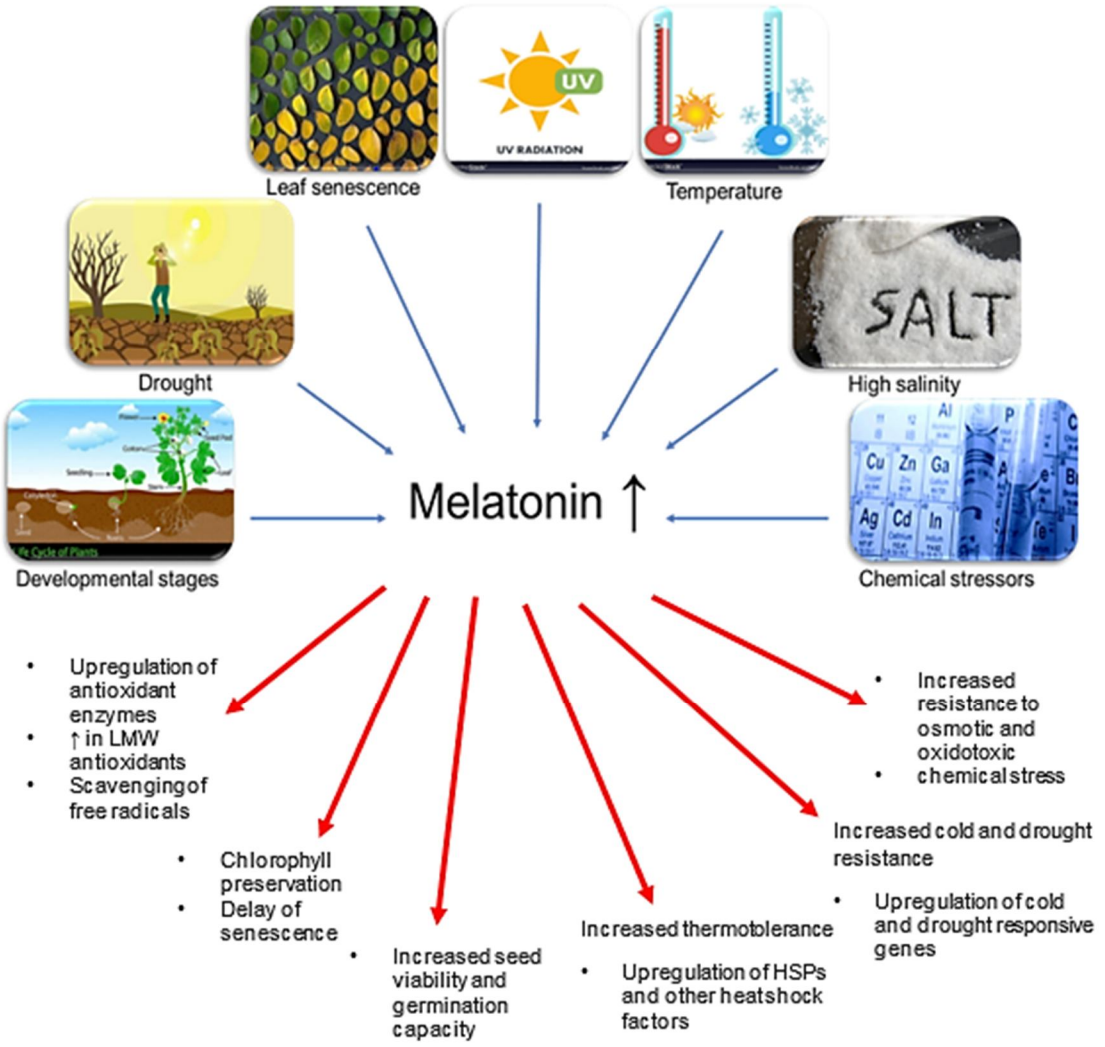

Figure 2.7: Increases in melatonin and their consequences for protection and stress tolerance. HSP, heat shock protein; LMW, low-molecular weight. Some actions may be species-specific or conditional. Adapted from Hardeland (2016).

2.5.3 Melatonin production by microorganisms and genetic manipulation of yeast to produce melatonin

Several melatonin producing yeast and bacterial species were identified recently although publications reporting melatonin production are limited (Table 2.3). All publications report very low levels of melatonin (often at the limit of detection) and show low and rather inconsistent production. In these studies, growth conditions had a significant influence on the levels of indoleamines synthesised by microorganisms. Sprenger et al. (1999) were the first group to report on the production of melatonin by *S. cerevisiae.* In this study, melatonin intracellular levels in the yeast dropped below detection level when cells were exposed to a salt medium. Melatonin and 5-methoxytryptamine levels recovered when these starved cells were resuspended in medium that contained tryptophan, serotonin or N-acetylserotonin. Rodriguez-Naranjo et al. (2012) found that tryptophan concentration increased the amount and rate at which melatonin was produced by *Saccharomyces* yeast strains and synthesis also depended on the medium composition. Vigentini et al (2015) found similar results in both *Saccharomyces* and non-*Saccharomyces* yeast strains. This study compared the ability of different yeast species to produce tryptophan derivatives in laboratory medium with two different concentrations of tryptophan and greater melatonin concentrations were found in the supernatant of minimal medium with 100 mg/L than 20 mg/L tryptophan.

The growth phase of yeast also seems to play a role in the levels of melatonin produced and studies thus far have shown that melatonin is mainly produced during the exponential growth phase (Rodriguez-Naranjo et al., 2012; Vigentini et al., 2015; Fernández-Cruz et al., 2017). Carbon source as well as oxygen availability did not seem to have a strong impact on the production pattern of melatonin in yeast. More recently, a genetically modified *S. cerevisiae* strain capable of forming tryptophan via the shikimic acid pathway was engineered to produce high quantities of melatonin by cloning genes encoding melatonin biosynthetic pathway enzymes of mammalian, trematodal and bacterial origin (Germann et al., 2016). These authors constructed the biosynthetic and recycling pathways for BH4 which is absent in yeast by overexpressing the *Rattus norvegicus* 6-pyruvoyl-tetrahydropterin synthase and sepiapterin reductase, 4a-hydroxytetrahydrobiopterin dehydratase (PCBD1) from *Lactobacillus ruminis* (Lr) or *Pseudomonas aeruginosa* (Pa) and 6-pyruvovyl-tetrahydropterin synthase (DHPR) from *Homo sapiens* (Hs) or *Rattus norvegicus* (Rn). To complete the biosynthetic pathway, they inserted TPH genes from *Schistosoma mansoni* (Sm) or *Homo sapiens*, *Homo sapiens* 5-hydroxy-L-tryptophan decarboxylase, *Bos taurus BtAANAT* and *Homo sapiens HsASMT*. When cultivated in mineral medium under aerobic conditions, strains carrying the PaPCBD1/RnDHPR recycling pathway produced more melatonin than those carrying the LrPCBD1/HsDHPR homologs. They also found that the SmTPH in the presence

of PaPCBD1/RnDHPR produced higher titres of melatonin and N-acetylserotonin in comparison to the HsTPH.

Light has an impact on the levels of melatonin produced by the photosynthetic bacterium *Erythrobacter longus.* Tilden et al. (1997) found that the melatonin levels in the cultures grown in complete darkness were significantly higher than the cultures grown under light conditions (Table 2.3). The study also showed that melatonin levels increased over time in the dark at a faster rate than the light grown cultures. Jiao et al. (2016) and Ma et al. (2017) showed that endophytic bacteria *Bacillus amyloliquefaciens SB-9, Bacillus thuringiensis CS-9, Agrobacterium tumefaciens CS-30, Pseudomonas fluorescens RG11*, isolated from grape vine roots could synthesise melatonin in aerobic conditions as well as promote abiotic stress induced production of melatonin in their hosts. The highest concentrations observed were produced 6 h after inoculation when cell density was low, thereafter concentrations declined with increasing cell density. These results show that growth phase may play a role in concentrations of melatonin produced by bacteria.

Table 2.3: Production of melatonin by microorganisms in laboratory media

Species	Medium	Sugar concentrations	Tryptophan concentrations	Growth conditions	Melatonin concentrations	Reference
Yeast						
S. cerevisiae (baker's yeast)	Yeast peptone glucose (YPD)	20 g/L glucose	-	Aerobic growth	9.3 - 94.7 ng/mg protein	(Sprenger et al., 1999)
	Salt medium	-	-	Aerobic growth	N/D	(Sprenger et al., 1999)
	Salt medium	-	0.204 g/L (1mM)	Aerobic growth	25.3 ng/mg protein	(Sprenger et al., 1999)
S. cerevisiae (Enoferm QA23)	YPD	20 g/L glucose	0.59 g/L	Aerobic growth	Present	(Rodriguez-Naranjo et al., 2012)
	Synthetic grape must (SGM)	20 g/L glucose/fructose	0.17 g/L	Aerobic growth	Present	(Rodriguez-Naranjo et al., 2012)
	SGM	20 g/L glucose/fructose	0.87 g/L	Aerobic growth	Present	(Rodriguez-Naranjo et al., 2012)
	SGM	200 g/L glucose/fructose	0.87 g/L	Anaerobic growth	Absent	(Rodriguez-Naranjo et al., 2012)
S. cerevisiae (Viniferm ARM)	SGM	20 g/L glucose/fructose	0.87 g/L	Aerobic growth	Present	(Rodriguez-Naranjo et al., 2012)
	SGM	200 g/L glucose/fructose	0.87 g/L	Anaerobic growth	Absent	(Rodriguez-Naranjo et al., 2012)
S. uvarum (Lalvin S6U)	SGM	20 g/L glucose/fructose	0.87 g/L	Aerobic growth	Present	(Rodriguez-Naranjo et al., 2012)
	SGM	200 g/L glucose/fructose	0.87 g/L	Anaerobic growth	Present	(Rodriguez-Naranjo et al., 2012)
S. cerevisiae var bayanus (Uvaferm BC)	SGM	200 g/L glucose/fructose	0.87 g/L	Anaerobic growth	Present	(Rodriguez-Naranjo et al., 2012)

Species	Medium	Sugar concentrations	Tryptophan concentrations	Growth conditions	Melatonin concentrations	Reference
S. cerevisiae (UMY255)	Yeast nitrogen base (YNB)	20 g/L glucose	100 mg/L	Aerobic growth	14.1 ng/10^9 cells	(Vigentini et al., 2015)
S. cerevisiae (EC1118)	YNB	20 g/L glucose	100 mg/L	Aerobic growth	0.16-0.37 ng/10^9 cells	(Vigentini et al. 2015
S. cerevisiae (IOC18-2007)	YNB	20 g/L glucose	100 mg/L	Aerobic growth	2.0 ng/10^9 cells	(Vigentini et al. 2015
Torulaspora delbrueckii UMY196	YNB	20 g/L glucose	100 mg/L	Aerobic growth	1.6 ng/10^9 cells	(Vigentini et al. 2015
T. delbrueckii UMY336	YNB	20 g/L glucose	100 mg/L	Aerobic growth	0.41-3.0 ng/10^9 cells	(Vigentini et al. 2015
T. delbrueckii CBS1146^T	YNB	20 g/L glucose	100 mg/L	Aerobic growth	2-9 ng/10^9 cells	(Vigentini et al. 2015
Zygosaccharomyces bailii ATCC36947^T	YNB	20 g/L glucose	100 mg/L	Aerobic growth	0.83-37.2 ng/10^9 cells	(Vigentini et al. 2015
Bacteria						
Erythrobacter longus	Shioi's medium	Glycerol enriched	N/A	Aerobic growth in the light	126 ± 28 pg/mg protein	(Tilden et al., 1997)
				Aerobic growth in the dark	9740 ± 2430 pg/mg protein	(Tilden et al., 1997)
Bacillus amyloliquefaciens SB-9	Nutrient broth (NB)	1 g/L glucose	200 mg/L	Aerobic growth	7.75 ng/10^{12} cells	(Jiao et al., 2016)
Bacillus thuringiensis CS-9	NB	1 g/L glucose	200 mg/L	Aerobic growth	3.33 ng/10^{12} cells	(Jiao et al., 2016)
Agrobacterium tumefaciens CS-30	NB	1 g/L glucose	200 mg/L	Aerobic growth	2.90 ng/10^{12} cells	(Jiao et al., 2016)
Pseudomonas fluorescens RG11	NB	1 g/L glucose	200 mg/L	Aerobic growth	1.32 ± 0.12 ng mL^{-1}	(Ma et al., 2017)
Oenococcus oeni UMB473	Synthetic wine	2.5 g/L glucose 2.5 g/L fructose	500 mg/L	Anaerobic growth in the dark	0.0013 ± 0.0002 ng/mL	(Fracassetti et al., 2020)
O. oeni UMB436	Synthetic wine	2.5 g/L glucose 2.5 g/L fructose	500 mg/L	Anaerobic growth in the dark	0.0022 ± 0.0008 ng/mL	(Fracassetti et al., 2020)

O. oeni UMB438	Synthetic wine	2.5 g/L glucose 2.5 g/L fructose	500 mg/L	Anaerobic growth in the dark	0.0027 ± 0.0004 ng/mL	(Fracassetti et al., 2020)
O. oeni UMB471	Synthetic wine	2.5 g/L glucose 2.5 g/L fructose	500 mg/L	Anaerobic growth in the dark	0.0026 ± 0.0009 ng/mL	(Fracassetti et al., 2020)
O. oeni UMB474	Synthetic wine	2.5 g/L glucose 2.5 g/L fructose	500 mg/L	Anaerobic growth in the dark	0.0021 ± 0.0005 ng/mL	(Fracassetti et al., 2020)
O. oeni UMB475	Synthetic wine	2.5 g/L glucose 2.5 g/L fructose	500 mg/L	Anaerobic growth in the dark	0.0025 ± 0.0013 ng/mL	(Fracassetti et al., 2020)
O. oeni UMB434	Synthetic wine	2.5 g/L glucose 2.5 g/L fructose	500 mg/L	Anaerobic growth in the dark	0.0036 ± 0.0007 ng/mL	(Fracassetti et al., 2020)
O. oeni UMB462	Synthetic wine	2.5 g/L glucose 2.5 g/L fructose	500 mg/L	Anaerobic growth in the dark	0.0049 ± 0.0010 ng/mL	(Fracassetti et al., 2020)
O. oeni UMB472	Synthetic wine	2.5 g/L glucose 2.5 g/L fructose	500 mg/L	Anaerobic growth in the dark	0.0078 ± 0.0023 ng/mL	(Fracassetti et al., 2020)
O. oeni UMB477	Synthetic wine	2.5 g/L glucose 2.5 g/L fructose	500 mg/L	Anaerobic growth in the dark	0.0049 ± 0.0007 ng/mL	(Fracassetti et al., 2020)

2.5.4 Putative melatonin functions in yeast

Studies on the role of melatonin in yeast physiology are expanding and recently three independent studies described the antioxidant role of the indoleamine in yeast. Melatonin was found to protect *S. cerevisiae* from hydrogen peroxide stress by increasing the expression of genes related to antioxidant defence such as catalase, superoxide dismutases, glutaredoxin, thioredoxin and the glutathione system (Vázquez et al., 2017; Bisquert et al., 2018). It also reduced the levels of oxidised glutathione and ROS accumulation in these studies. Vázquez et al. (2018) investigated the impact of melatonin on various *Saccharomyces* and non-*Saccharomyces* yeast strains challenged with H_2O_2 and found that melatonin supplementation reduced ROS accumulation and lipid peroxidation in all evaluated strains. They also found that melatonin supplementation increased oleic and palmitoleic acids in cell membranes resulting in higher unsaturated fatty acid to saturated fatty acid ratios which were previously linked to higher H_2O_2 tolerance. The ability for melatonin to act as a yeast signalling molecule during wine fermentation was investigated by Valera et al (2019). Although melatonin supplementation did not have an impact on yeast biomass, fermentations kinetics in the mixed culture fermentations were faster than the control and non-*Saccharomyces* species persisted until the end of fermentation in some cases. However, the changes observed were dependent on the nitrogen levels in the media and the composition of the inoculum. Further studies are required to determine if melatonin has signalling abilities in yeast but the high concentrations of melatonin (0.5-1 g/L) used in this study are uncharacteristic of a signalling molecule.

2.6 Conclusions

Since the discovery of melatonin in the 1950's, our understanding on the biosynthetic pathways and physiological functions in all living organism keeps expanding. This indoleamine has a plethora of functions in plants and animals and recently studies suggest that multiple biosynthetic pathways may exist in all kingdoms. In the last decade, studies on the production of melatonin by yeast have advanced significantly but production patterns, biosynthetic pathways and enzymes involved in these pathways still needs to be elucidated. Physiological studies in yeast thus far have focused on H_2O_2 induced stress but yeast cultures are exposed to various environmental stressors and the impact of melatonin on these stressors needs to be investigated further.

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Chapter 3

Characterization of melatonin production by yeast – an *in vitro* and *in silico* study

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3.1 Abstract

The synthesis and biological role of melatonin in microorganisms remains largely unknown. Recent studies have shown that yeast produce this metabolite in several conditions including fermentation, starvation and in some cases respiration. These studies highlighted the importance of tryptophan as a precursor molecule, but also revealed inconsistencies in the production patterns of this metabolite. In this study, we evaluated the impact of several growth conditions on melatonin formation in wine-associated yeast species. Conditions included changes to nutrient composition, the addition of biosynthetic pathway intermediates and the application of environmental stressors. The data showed that production, if present, was always at very low levels, but the concentrations varied significantly including between biological repeats and no single setting or condition led to reproducible melatonin formation. Analysis of precursors nevertheless suggest that the biosynthesis pathway in yeast may involve 5-hydroxytryptophan and serotonin as intermediates. An *in silico* screen of available genome sequences of yeast also did not reveal any orthologs of plant or animal melatonin biosynthesis genes. Taken together, the data suggests that standard growth conditions and environmental stress assays do not elicit a reproducible production of melatonin. The role of melatonin might be linked to environmental conditions that are not easily reproduced in a laboratory setting, or the compound might oscillate and only be produced fleetingly. The irreproducible nature and low level of melatonin production could also suggest that melatonin might be an overflow metabolite in yeast, a hypothesis also supported by published transcriptomic data that suggest only very minor and non-specific impacts of melatonin on gene transcription in laboratory growth conditions.

Key words: *Saccharomyces cerevisiae*, *Kazachstania spp*, melatonin, biosynthesis, indolic compounds.

3.2 Introduction

Melatonin (MEL) is an indolic compound synthesised from the amino acid tryptophan which was originally isolated from bovine pineal gland (Lerner et al., 1958) and thus incorrectly classified as a neurohomone. Since this discovery, several studies have shown that this indoleamine is synthesised in other organs in vertebrates as well in other organisms such as invertebrates, plants, bacteria and macroalga (Pandi-Perumal et al., 2006). The biosynthetic

pathway of MEL in animals and plants has been elucidated and involves four enzymatic steps. The eight enzymes that have been found to be involved in this pathway include tryptophan hydroxylase (TPH), aromatic L-amino acid decarboxylase (AADC), arylalkylamine N-acetyltransferase (AANAT), serotonin N-acetyltransferase (SNAT), N-acetylserotonin methyltransferase (ASMT), hydroxyindole-O-methyltransferase (HIOMT), tryptamine 5-hydroxylase (T5H), and tryptophan decarboxylase (TDC). This pathway also involves several intermediates including 5-hydroxytryptophan (5-HTP), tryptamine, serotonin (5-HT) and 5-methoxytryptamine (5-MT), 6-hydroxymelatonin (6-OHM), 3-indole acetic acid (3-IAA) and tryptophol (IEA) (Axelrod and Weissbach, 1960).

In animals, the main regulator of MEL secretion from the pineal gland is the circadian clock resulting in low concentrations in human serum during the day and night time peak concentrations (Pevet and Challet, 2011). Diurnal changes in MEL levels were also observed in photosynthetic *Rhodospirillum rubrum* and it is hypothesised that the low levels during the photophase might be caused by increased utilization rather than a decrease in the rate of synthesis (Manchester et al., 1995; Tilden et al., 1997). Roopin et al. (2013) observed a diel rhythm of MEL production in the dinoflagellate *Symbiodinuim*. In this study, MEL levels peak during the dark and lowest levels were recorded during the day, but this pattern was lost when the organism was kept in constant darkness. The authors hypothesised that the oscillating pattern of MEL in this organism is a result of increased photo-consumption by free radicals during the day rather than an endogenous circadian rhythm. MEL production in plants is not regulated by circadian rhythm in the same manner observed in vertebrates. In plants, MEL production is stimulated by exposure to light when larger quantities of reactive oxygen species (ROS) are generated by photosynthesis (Tan et al., 2012; Reiter et al., 2015).

MEL functions as a free radical scavenger and antioxidant in all organisms. In addition to circadian regulation, studies have shown that MEL production is enhanced by exposure to biotic and abiotic stresses in various organisms. High temperatures increased MEL synthesis in rice seedlings by increasing activities of SNAT and ASMT (Byeon and Back, 2014) whereas infection of *Arabidopsis thaliana* with the avirulent pathogen *Pseudomonas syringae* DC3000 induced MEL synthesis in the plant (Lee and Back, 2017). Exposure to cold stress resulted in increased production of MEL in the microalgae *Gonyaulax polyedra* (Fuhrberg et al., 1997).

The first report of MEL production by *Saccharomyces cerevisiae* was made by Sprenger et al. (1999). In this study, the authors found that following starvation period in salt medium, re-incubation of cultures in growth medium supplemented with tryptophan, 5-HT or *N*-acetylserotonin increased MEL and 5-MT concentrations. Rodriguez-Naranjo et al. (2012) found that production of MEL was dependent on tryptophan levels, concentrations of reducing

sugars and growth phase of yeast. Building on this work, Vigentini et al. (2015) confirmed the role of tryptophan in MEL production in *Saccharomyces* and non-*Saccharomyces* yeast species in a strain dependent manner when cultured in laboratory medium. However, none of the yeast synthesised MEL under oenological conditions. More recently, Fernández-Cruz et al. (2017) investigated the production pattern of MEL and pathway intermediates of MEL in *Saccharomyces* and non-*Saccharomyces* yeasts during alcoholic fermentation. In the latter study, the occurrence of MEL was variable and the precursor compounds of MEL, i.e. 5-HT and *N*-acetylserotonin were barely quantifiable during fermentation.

MEL production patterns by yeast documented thus far are therefore inconsistent. This study aimed to characterise MEL production in yeast in response to factors such as nutrient composition, environmental stress and supplementation with MEL pathway intermediates. In addition, *in silico* sequence analysis to identify potential MEL biosynthesis enzymes was carried out. To the best of our knowledge, this is the first study that tested a wide array of conditions to characterise MEL production in yeast.

3.3 Materials and method

3.3.1 Screening of yeast isolates for melatonin production

Yeast strains used in this study were obtained from the culture collection of the South African Grape and Wine Research Institute (IWBT) at Stellenbosch University, Westerdijk Fungal Biodiversity Institute (CBS) collection, Lallemand Inc, Fermentis Lesaffre, Erbsloh Geisenheim AG and Anchor Yeast (Table 3.1). IWBT cultures were isolated from grape cultivars in the Stellenbosch region. The yeast stock cultures were maintained in 20% (v/v) glycerol at -80°C and were streaked out on Wallerstein Laboratory Nutrient agar (Sigma-Aldrich, Spain), when required. The plates were incubated at 30°C for 3-5 days.

Table 3.1: Yeast strains screened for MEL production

Yeast species	Strain number	Yeast species	Strain number
Aureobasidium pullulans	IWBT Y1011 [a]	*Metschnikowia pulcherrima*	IWBT Y1094 [a]
Aureobasidium pullulans	IWBT Y1068 [a]	*Metschnikowia pulcherrima*	IWBT Y1103 [a]
Cryptococcus flavescens	IWBT Y1006 [a]	*Metschnikowia pulcherrima*	IWBT Y1119 [a]
Cryptococcus flavescens	IWBT Y844 [a]	*Metschnikowia pulcherrima*	IWBT Y1118 [a]
Cryptococcus oriensis	IWBT Y872 [a]	*Metschnikowia pulcherrima*	IWBT Y1425 [a]
Hanseniaspora opuntiae	IWBT Y1043[a]	*Metschnikowia pulcherrima/fructicola*	IWBT Y1005 [a]
Hanseniaspora opuntiae	IWBT Y1055 [a]	*Pichia caribbica*	IWBT Y852 [a]
Hanseniaspora opuntiae	IWBT Y1085 [a]	*Pichia carribica*	IWBT Y1036 [a]
Hanseniaspora opuntiae	IWBT Y875 [a]	*Pichia guilliermondi*	IWBT Y853 [a]
Hanseniaspora opuntiae/uvarum	IWBT Y1056 [a]	*Saccharomyces bayanus*	VR44 [d]
Hanseniaspora uvarum	IWBT Y1044 [a]	*Saccharomyces bayanus*	CLIB 2025[g]
Hanseniaspora uvarum	IWBT Y1092 [a]	*Saccharomyces bayanus*	BC5 103[g]
Hanseniaspora uvarum	IWBT Y1096 [a]	*Saccharomyces bayanus*	Oenferm bouquet [e]
Hanseniaspora uvarum	IWBT Y1097 [a]	*Saccharomyces cerevisiae*	IWBT Y1022 [a]
Hanseniaspora uvarum	IWBT Y1098 [a]	*Saccharomyces cerevisiae*	IWBT Y1024 [a]
Hanseniaspora uvarum	IWBT Y1100 [a]	*Saccharomyces cerevisiae*	IWBT Y1052 [a]
Hanseniaspora uvarum	IWBT Y1104 [a]	*Saccharomyces cerevisiae*	IWBT Y1080 [a]
Hanseniaspora uvarum	IWBT Y1116 [a]	*Saccharomyces cerevisiae*	IWBT Y1088 [a]
Hanseniaspora uvarum	IWBT Y1117 [a]	*Saccharomyces cerevisiae*	IWBT Y829 [a]
Hanseniaspora uvarum	IWBT Y1121 [a]	*Saccharomyces cerevisiae*	IWBT Y915 [a]
Hanseniaspora uvarum	IWBT Y1131 [a]	*Saccharomyces cerevisiae*	IWBT Y911 [a]
Hanseniaspora uvarum	IWBT Y1135 [a]	*Saccharomyces cerevisiae*	IWBT AL 146 [a]
Hanseniaspora uvarum	IWBT Y1138 [a]	*Saccharomyces cerevisiae*	IWBT Y858 [a]
Hanseniaspora uvarum/clermontiae	IWBT Y1059 [a]	*Saccharomyces cerevisiae*	IWBT Y913 [a]
Hanseniaspora vineae	IWBT Y1021 [a]	*Saccharomyces cerevisiae*	IWBT Y805 [a]
Hanseniaspora vineae	IWBT Y1034 [a]	*Saccharomyces cerevisiae*	AL171 [a]
Hanseniaspora vineae	IWBT Y1090 [a]	*Saccharomyces cerevisiae*	BM45 [c]
Hanseniaspora opuntiae	IWBT Y1101 [a]	*Saccharomyces cerevisiae*	VIN13[a]
Issatchenkia orientalis	IWBT Y1130 [a]	*Saccharomyces cerevisiae*	EC1118 [c]
Kazachstania aerobia	CBS 9918^{T} [b]	*Saccharomyces cerevisiae*	Cross evolution [c]
Kazachstania hellenica	CBS 10706^{T} [b]	*Saccharomyces cerevisiae*	QA23 [c]
Kazachstania servazii	CBS 4311^{T} [b]	*Saccharomyces cerevisiae*	IWBT Y1422 [a]
Kazachstania solicola	CBS 6904^{T} [b]	*Saccharomyces cerevisiae-flocculate*	IWBT Y1050 [a]
Lachancea thermotolerans	IWBT Y1109 [a]	*Tremella globispora*	IWBT Y1009 [a]
Metschnikowia fructicola	IWBT Y1423 [a]	*Tremella globispora*	IWBT Y1081 [a]
Metschnikowia pulcherrima	IWBT Y1093 [a]	*Wickerhamomyces anomalus*	IWBT Y934 [a]
Metschnikowia pulcherrima	IWBT Y1093 [a]	*Zygosaccharomyces bailii*	IWBT Y874 [a]

[a] South African Grape and Wine Research Institute (IWBT) collection
[b] Westerdijk Fungal Biodiversity Institute (CBS) collection
[c] Lallemand
[d] Fermentis Lesaffre
[e] Erbsloh Geisenheim AG
[f] Anchor Yeast
[g] Unknown source

3.3.1 Media and growth conditions

Experiments in this study were conducted in the following medium: Synthetic grape juice (SGJ) used was composed of (per litre) varying amounts of sugars, 0.46 g NH_4Cl, 0.2 g citric acid, 3 g malic acid, 2.5 g KH tartrate, 1.23 g $MgSO_4.7H_2O$, 1.14 g K_2HPO_4, pH 3.3, 100 mg alanine, 750 mg arginine, 50 mg asparagine, 350 mg aspartic acid, 200 mg glutamine, 500 mg glutamic acid, 50 mg glycine, 150 mg histidine, 200 mg isoleucine, 300 mg leucine, 250 mg lysine, 150 mg methionine, 150 mg phenylalanine, 500 mg proline, 400 mg serine, 350 mg threonine, 179 mg tryptophan, 20 mg tyrosine, 200 mg valine and anaerobic factors (ergosterol 10 mg, Tween 80 0.5 mL) (Henschke and Jiranek, 1993); minimal medium (MM) (per litre: 20 g or 80 g glucose; 6.7 g yeast nitrogen base (YNB; Difco, Heidelberg, Germany), pH 5.4); yeast peptone dextrose medium (YPD) (per litre: 20 g dextrose, 20 g peptone, 10 g yeast extract)

SGJ experiments were performed in 100 mL spice jars covered with a rubber stopper with a S-shaped fermentation airlock and filled with 50 mL SGJ in duplicate. Cultures were inoculated to an initial optical density (OD_{600}) of 0.1 for the *Saccharomyces* strains and 0.2 for all the non-*Saccharomyces* strains. Under fermentative conditions, non-saccharomyces strains had a slower growth and therefore were inoculated at a higher optical density to ensure more biomass at the 48h sampling point. Fermentations were carried out at 30°C without agitation. Samples were taken on the second day of fermentation and centrifuged at 12000 rpm for 3 min. A 1 mL volume of the supernatant was analysed using LC-MS/MS for detection and quantification of MEL. Depending on the experiment, the standard SGJ was modified by addition of different concentrations of glucose (20 and 200 g/L) and adjusting tryptophan concentrations to levels used by Rodriguez-Naranjo et al. (2012) (0.17, and 0.87 g/L). Inoculated cultures were grown under the following conditions: two oxygen limited conditions (50 mL in 100 mL spice jars with gas traps without agitation and 200 mL in 250 mL flasks with agitation at 120 rpm) and aerobic conditions (25 mL in 250 mL flasks with agitation at 120 rpm). Samples were taken after 48 h growth for analysis of MEL in the supernatant. Experiments were done in at least duplicate.

The experimental layout for the analysis of MEL production in defined medium, MM was adapted from Vigentini et al. (2015). Yeast strains were inoculated in 5 mL YPD and cultivated at 25°C for 24 h on a test tube rotor wheel. Once cultures reached approximately 2–4 OD_{600}, cells were centrifuged at 6000 g for 10 min and washed once in sterile distilled H_2O. Pellets were resuspended in 10 mL MM for 24 h to allow nutrients depletion. Starved yeasts were centrifuged at 4000 rpm for 10 min and resuspended in 2 mL sterile distilled H_2O. Strains were cultivated in MM containing 20 g/L glucose supplemented with either 20 mg/L tryptophan or 100 mg/L tryptophan as precursor. Growth tests were performed in 6-well sterile

plates with 10 mL growth medium at 25˚C without shaking. The initial OD_{600} of inoculum was approximately 0.2 per well. Plates were covered with a sterile lid to avoid any possible cross-contamination. Experiments continued for three days with sampling at 12, 24, 48 and 72 h after the inoculation.

To assess the impact of oxygen limitation on MEL production, strains were cultivated in MM containing 20 g/L glucose and 20 mg/L tryptophan as precursor. Growth tests were performed in 250 mL Erlenmeyer flasks and contained 100 mL growth medium. Flasks were covered with a rubber stopper with a S-shaped fermentation airlock and incubated at 28°C with agitation at 110 rpm or without agitation. The initial OD_{600} of inoculum was approximately 0.2 per flask. Cell counts on cultures were carried out by taking 0.1 mL aliquots of appropriate decimal dilutions in sterile saline water and spreading on YPD agar plates. Colonies were enumerated after incubation at 25°C for 3–5 days, depending on the species. Experiments were carried out in triplicate.

3.3.2 Melatonin pathway intermediates

S. cerevisiae IWBT Y805 was precultured and cultivated in MM containing 20 g/L glucose and 20 or 100 mg/L TRP as precursor. Growth tests were performed in 250 mL Erlenmeyer flasks containing 100 mL growth medium and incubated at 30°C with agitation at 120 rpm. The initial OD_{600} of inoculum was approximately 0.2 per flask. Flasks were covered with foil and closed with a cotton cap. In some experiments, 50 µM final concentration tetrahydrobiopterin (BH4), a cofactor in the animal MEL synthesis pathway (Fig 2.2) was added to MM while 5-HT and 5-HTP was supplemented to a 1 mM final concentration. Cultures were grown at 30°C and samples were taken at 0, 30 min, 2 h, 4 h and 6 h for LC-MS analysis. Experiments were performed in triplicate.

3.3.3 Carbon sources experiments

Four carbon sources were evaluated for influence on MEL production. *S. cerevisiae* was precultured in YPD until mid-log phase (OD_{600} of 2-4) and then washed once with sterile 0.9% NaCl saline solution. Cultures were re-inoculated 10 mL MM without carbon source and incubated at 30°C for 2 h for carbon source depletion. Cultures were then re-inoculated in 60 mL MM with 2% carbon source (20 g/L glucose, 20 g/L galactose, 10 g/L glycerol + 10 g/L ethanol or 20 g/L maltose) to an OD_{600} of 0.2 and grown at 30°C with agitation. Flasks were covered with foil and closed with a cotton cap. Samples were taken for LC-MS analysis at 0, 30 min and 2, 4, 6 and 12 h after re-inoculation into MM with selected carbon sources and growth absorbance was measured. Experiments were performed in triplicate.

3.3.4 Environmental stress experiments

Four stress compounds (3.5 mM H_2O_2, 2.5 mM $ZnCl_2$, 5 mM $CuSO_4$, 0.4 mM NaCl) and various pH values (3, 5, 7 and 9) were evaluated. Yeast strains were inoculated in 5 mL YPD medium and incubated at 28°C on a test tube rotator wheel. Overnight cultures were centrifuged at 12000 *g* for 3 min and washed once in sterile distilled H_2O. Yeasts were re-inoculated into 100 ml YPD to an OD_{600} of 0.2 and grown at 28°C with agitation at 120 rpm. Stressors were added at the start of the experiment after the addition of the culture and growth absorbance and MEL levels were measured in samples over 24 h. In order to determine whether stressors have an impact during exponential growth, the yeast cells were prepared as described above and allowed to grow for 5 h until the start of exponential growth. The stressors were added at this point and samples were taken at 0 min, 30 min 1 h and 2 h. Experiments were performed in triplicate.

3.3.5 Characterisation of melatonin production in nitrogen limited continuous culture conditions

Strain IWBT Y805 was precultured overnight MM with 5 g/L $(NH_4)_2SO_4$. The preculture was inoculated into 600 mL MM [80 g/L glucose; 1.7 g/L YNB without amino acids (Difco, Heidelberg, Germany); pH 4.2] with different concentrations of nitrogen (described in 3.3.5.1 below) to an initial OD_{600} of 0.2 in 1L BioFlo® 110 reactor (New Brunswick Scientific Enfield, CT). Cultures were grown at 25°C with agitation at 300 rpm. Sterile compressed air was sparged into the bioreactor at a constant flow rate of 0.5 vvm with the dissolved oxygen tension maintained above 50% of saturation. Growth was monitored spectrophotometrically, and experiments were carried out in duplicate.

The dilution rate in each experimental condition was determined from the growth rate of the cultures in MM. This was calculated from OD measurements taken during exponential growth phase in batch culture where OD_0 is initial OD and OD_t is the OD at time *t.*

$$D = \ln (OD_t/OD_0)$$

The continuous cultures were grown for four residence times to achieve steady state, which was confirmed by stable absorbance readings at 600 nm. Once steady state was reached, samples were collected. Extracellular concentrations of glucose and ammonia and intra- and extracellular samples concentrations of TRP, 5-HTP, 5-HT, 5-MT, MEL, 6-OHM, 3-IAA and IEA were determined.

3.3.5.1 Tryptophan supplementation in nitrogen limited continuous culture fermentation

MM was supplemented with 0.3, 0.6 and 5 g/L $(NH_4)_2SO_4$ to determine the limiting nitrogen concentration in batch conditions. Nitrogen limited concentrations determined under batch conditions were confirmed in continuous culture. Once steady state was achieved in the nitrogen limited continuous culture, TRP was sterilely injected to feed reservoir or directly to the bioreactor to give an initial bioreactor concentration of 50 mg/L. Samples were taken before addition of TRP, immediately after TRP addition, 5 min and then every 30 min for 3 h.

3.3.5.2 Tryptophan as sole nitrogen source

Cultures were cultivated under the same conditions described above with the following modification. $(NH_4)_2SO_4$ was replaced with 5 mM TRP (140 mg N/L) as a sole nitrogen source, a level double to the nitrogen level supplied by $(NH_4)_2SO_4$. Once steady state was confirmed, samples were taken in three-hour intervals over a 9 h period.

3.3.5.3 Impact of light/dark cycle entrainment

Cultures were cultivated in MM containing 80 g/L glucose with 0.2 g/L $(NH_4)_2SO_4$ (42.4 mg N/L) and 100 mg/L TRP (13.7 mg N/L). Experimental conditions were established by feeding the bioreactor with MM and after approximately 46 h of batch cultivation. Once steady state was confirmed, cultures were conditioned to an artificial light/dark cycle for 3 days. LED white lights (~200 µmol $m^{-2}s^{-1}$ (9W bulb)) were used to create 12 h light cycles. Aluminium foil was wrapped around the bioreactor to create darkness for another 12 h. Samples were taken twice in the light (4 pm and 7 pm) and dark phases (4 am and 7 am) for analysis of metabolites.

3.3.6 Metabolite Extraction

Intracellular MEL was extracted from 1 mL culture centrifuged at 7840 rpm for 3 min at 4ºC. The cells were washed once with saline solution and resuspended in 500 µL boiling buffered ethanol (75% Ethanol, 10mM HEPES pH 7.0) followed by incubation for 3 min at 95ºC. The cell debris was collected by centrifugation at 15000 rpm for 1 min and the supernatant was transferred to a new 1.5 mL centrifuge tube. The ethanol was evaporated under vacuum and the remaining pellet was resuspended in 500 µL of a 15% (v/v) methanol solution. Methanol insoluble material was pelleted by centrifugation at room temperature for 6 min at 12000 rpm. The supernatant was transferred to a clean tube and diluted with 500 µL acetonitrile. Extracellular MEL concentration was determined from 1 mL of culture centrifuged at 12000

rpm for 3 min at room temperature to remove the cells. The intracellular and extracellular samples were analysed by UPLC-MS.

3.3.7 Liquid chromatography-mass spectroscopy (LC-MS/MS) analysis

TRP, 5-HTP, 5HT, 5-MT, MEL, 6-OHM, 3-IAA and IEA external standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). The LC-MS/MS analysis was carried out on an Acquity UPLC separation module (Waters, Milford, MA, USA) coupled with a Xevo TQ MS (Waters) triple quadrupole mass spectrometer. A Waters BEH C 18 column (100 x 2.1 mm, 1.7 µm) was used for separation. The mobile phase solvents were 0.1% formic acid (A) and acetonitrile (B). Separation was performed in gradient mode using the following elution profile: 5–60% (A in B) in 6 min and then 60–100% (A in B) in 3 min at a flowrate of 0.45 mL/min. The column and samples were kept at 40°C and 20°C, respectively. The capillary voltage was set to 3.0 kV, the cone voltage was 14 V, the source temperature was 130°C, the desolvating temperature was 350°C and the desolvation gas (nitrogen) was at 800 L/h. Argon was used as collision gas. All metabolites were determined in multiple reaction monitoring (MRM) mode, and the data were acquired by MassLynx 4.0 software (Waters). The mass spectrometer operated in the electrospray ionization (ESI) positive mode and the fragmentation transitions are described in Table 3.2. To keep the cone clean, the eluate from the first 1.1 min analysis was discarded by the divert valve. External standards were used to quantify all metabolites. Stock solutions were serially diluted in methanol to generate calibrators at eight concentration levels.

Table 3.2: Fragment transitions of metabolites analysed by LCMS/MS. Analysis was done on the Waters Acuity UPLC separation module.

Compound	Parent ion (*m/z*)	Daughter ion (*m/z*)	Cone voltage (v)	Collision energy (eV)
Tryptophan	205	118	14	16
		146		18
		188		18
5-hydroytryptophan	221	134	15	30
		162		15
		204		20
Serotonin	177	132	15	20
		160		
Melatonin	233.2	131	15	16
		159		25
		174.1		20
6-Hydroxymelatonin	249	189.9	15	20

3-Indole acetic acid	176.2	76.9 103 130	20	35 30 15
5-Methoxytryptamine	174	130 143 159	20	30 20 20
Tryptophol	162.2	117 144	15	20 15

3.3.8 *In silico* analysis to identify yeast orthologs of genes involved in melatonin biosynthesis

Enzymes involved in biosynthesis of MEL in plants and animals have been characterised (Figs 2.3 and2.4). Protein sequences of these enzymes namely TPH, AADC, AANAT, SNAT), ASMT, HIOMT, T5H, and TDC were used to search for orthologs in yeast genome. Consensus patterns of conserved domains of these proteins were generated on PROSITE (Sigrist et al., 2002, 2013) and these patterns were used to search for sequence matches in yeast.

3.4 Results

3.4.1 Media and experimental conditions evaluated

Several conditions were tested to determine melatonin production (Table 3.3). These included varying laboratory medium composition, supplementing media with different TRP concentrations and MEL biosynthetic pathway intermediates; adding environmental stressors immediately after inoculation or to cultures in exponential growth and varying growth conditions by increasing or decreasing oxygen availability and entraining cultures to a light/dark cycle. The limit of detection (LOD) of the mass spectrometer used in this study for all metabolites was 0.01 ng/mL. These were lower than the LOD reported in other studies where concentrations of metabolites (0.13, 30.16 and 0.034 ng/mL) were determined by HPLC-MS/MS (Rodriguez-Naranjo et al., 2011; Gomez et al., 2012; Kocadağli et al., 2014 respectfully). This low LOD confirmed the sensitivity of method to analyse the metabolites investigated in this study.

Table 3.3: Conditions tested to determine MEL production patterns by yeast.

Base media	Modification	Conditions tested	Sampling points	Cultures tested
Synthetic grape juice (SGJ)	SGJ with 100 g/L glucose and 100 g/L fructose.	Standard oenological fermentation.	48 h	All yeast strain listed in Table 3.1
	20 and 200 g/L glucose 0.17 and 0.87 g/L TRP	Oxygenation levels and agitation	48 h	*Kazachstania* spp and *S. cerevisiae* spp
Yeast peptone dextrose (YPD)	Environmental stress 3.5 mM H_2O_2, 2.5 mM $ZnCl_2$, 5 mM $CuSO_4$ and 0.4 mM NaCl	Stressors added after inoculation	0, 4, 7, 10, 12 and 24 h	*S. cerevisiae* IWBT Y805
		Stressors added in exponential growth	0, 0.5, 1 and 2 h	
Minimal medium (MM)	20 and 100 mg/L TRP 20 g/L glucose	Oxygenation levels and agitation	12, 24, 48 and 72 h	*S. cerevisiae* IWBT Y805
	No modification	MEL pathway intermediates and co-factor: BH4, 5-HT and 5-HTP	0, 0.5, 2, 4 and 6 h	
	Carbon source: 20 g/L galactose, 20 g/L maltose, 10 g/L glycerol + 10 g/L ethanol	Carbon source	0, 0.5, 2, 4, 6 and 12 h	
Minimal medium (Continuous culture)	80 g/L glucose 0.3 g/L $(NH_4)_2SO_4$	TRP pulse to steady state cultures • Injected directly into bioreactor once • Added to feed reservoir	Before addition of TRP Immediately after TRP addition; 5 min after; every 30 min for 6 h	*S. cerevisiae* IWBT Y805
	80 g/L glucose 5 mM TRP	TRP as sole nitrogen source	Every 3 h for 12 h	
	80 g/L glucose 0.2 g/L $(NH_4)_2SO_4$ 100 mg/L TRP	Light/dark cycle	Every 3 h for 12 h	

3.4.2 Screening of melatonin production by yeast

The initial dataset of 74 strains (Table 3.1) in SGJ (100 g/L glucose and 100 g/L fructose) reproduced standard oenological conditions. MEL was detected in media of eleven of these strains, with each strain representing a different species belonging to six genera. However, production patterns were inconsistent in all strains and in most experiments, melatonin was detectable but not always quantifiable (Table 3.4). The data was reported as area under peak in the chromatogram to show all the strains which produced detectable levels of melatonin. In

this initial screening, biomass data was not collected. Therefore, when quantified, levels of MEL could not be normalised to biomass levels at 48 h. Initially among the MEL producing strains, the highest levels based on peak area was found in *Kazachstania* species. However, in two follow-up experiments quantities of MEL produced by the *Kazachstania* species varied significantly. The metabolite was detected more frequently (in five out of six experiments) in the *S. cerevisiae* strain IWBT Y1080. Differences in production were observed in *Hanseniaspora* spp and *Pichia* spp. In the case of *Hanseniaspora* spp, MEL was detected in five of the six experiments with *H. opuntiae* (IWBT Y1101) whereas with *H. uvarum* (IWBT Y1104) and *H. vineae* (IWBT Y1090) strains it was detected in four of the six experiments. Although MEL was detected in both *Pichia* spp, the peak areas of *P. guilliermondi* (IWBT Y853) in most instances was greater than that of *P. caribbica* (IWBT Y852). MEL was detected least frequently in the *Z. baili* (IWBT Y874) strain.

Table 3.4: MEL produced by yeast strains in SGJ (100 g/L glucose and 100 g/L fructose) in duplicate replicates after 48-h fermentation expressed as area under the peak in the *UPLC-MS/MS* chromatogram

Species	Melatonin production (Area under peak the peak in the UPLC-MS/MS chromatogram)						
	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Experiment 6	Experiment 7
S. cerevisiae (IWBT Y1080)	39.7 nd [a]	138.8 3959	37.6 nd	nd nd	133.6 3967	194.29 207.50	Not tested
H. uvarum (IWBT Y1104)	128.4 1189	nd nd	17.1 nd	186.2 1286	nd 95.5	nd nd	Not tested
H. vineae (IWBT Y1090)	646 479	nd 33.2	nd nd	510 582	96.8 120.7	nd nd	Not tested
H. opuntiae (IWBT Y1101)	279 1276	139.7 nd	nd 20.8	161.4 298	96.2 185.1	nd nd	Not tested
Z. bailii (IWBT Y874)	2179 869	nd nd	nd nd	2668 1388	nd nd	nd nd	6.455 5.853 5.251
P. caribbica (IWBT Y852)	2210 494	nd nd	13.1 nd	2217 461	159.8 nd	nd nd	26.8 4.1 nd
P. guilliermondi (IWBT Y853)	170 15498	nd 31.2	nd nd	202 19915	113.9 109.3	nd nd	7.4 23.0 19.2
W. anomalus (IWBT Y934)	7079 169.6	nd nd	21.9 nd	1067 126.3	nd nd	nd nd	0.92 14.6 3.1
K. aerobia (CBS 9918)	5576[b]	6644 3703	47050[b]	Not tested	Not tested	Not tested	Not tested
K. servazii (CBS 4311)	333[b]	85021 101312	5066[b]	Not tested	Not tested	Not tested	Not tested
K. solicola (CBS 6904)	24645236[b]	21113288 704429	149154990[b]	Not tested	Not tested	Not tested	Not tested

[a] not detected
[b] single replicate

In follow up experiments, it was decided to investigate *Kazachstania* species in more detail. In addition, 20 *S. cerevisiae* strains (Table 3.1) were screened. Six strains were initially found to synthesise MEL in SGJ containing 200 g/L glucose under oxygen limited conditions, albeit again at very low concentrations (Table 3.5). Variations between biological repeats were again observed in this experiment, as MEL was usually detected and quantified in the supernatant samples of one of the biological repeats of each *S. cerevisiae* strain. Since the levels detected in these cases were significantly above the detection threshold of the method, these variations appear to be true reflections of MEL biosynthesis. The analysis nevertheless suggested that *Kazachstania* spp. indeed produced considerably higher levels of MEL compared to *S. cerevisiae* strains.

Table 3.5: Concentrations of extracellular and intracellular MEL (duplicate biological determinations) detected in SGJ (200 g/L glucose, 179 mg/L TRP) when strains were cultivated under oxygen limited conditions for 48 h.

Species	MEL concentration µg/L			
	Extracellular		Intracellular	
	Biological 1	Biological 2	Biological 1	Biological 2
K. hellinica (CBS 10706)	443.2	504.6	Not analysed	Not analysed
K. aerobia (CBS 9918)	426.3	493.5	0.71	1.90
K. servazii (CBS 4311)	467.3	564.3	0.59	1.43
*K. servazii (*CBS 4311)	491.9	512.1	Not analysed	Not analysed
K. solicola (CBS 6904)	0.2	nd[a]	0.81	0.59
S. cerevisiae (IWBT Y915)	0.01	Nd	0.05	0.13
S. cerevisiae (IWBT Y911)	0.01	Nd	0.83	0.79
S. cerevisiae (IWBT AL146)	0.01	Nd	Not analysed	Not analysed
S. cerevisiae (IWBT Y858)	0.01	Nd	1.23	0.73
S. cerevisiae (IWBT Y913)	0.13	Nd	0.92	1.20
S. cerevisiae (IWBT Y805)	0.99	Nd	0.67	1.19

[a]not detected

Intracellular concentrations of MEL from the cultures used in this experiment, with exception of *K. hellinica* and *S. cerevisiae* IWBT AL146 were also analysed. Most of the cultures (except *S. cerevisiae* IWBT Y915) revealed MEL levels of approximately 1 µg/L (Table 3.5) in all samples even though concentrations in the supernatant differed significantly. Follow-up experiments (four) to confirm intracellular concentrations in SGJ were contradictory and MEL was not detected in any of the samples.

3.4.3 The influence of medium composition, tryptophan concentrations and growth conditions on melatonin production

Since the previous data suggested that MEL production was highly inconsistent and did not provide clarity on production patterns or suggest any physiological roles, the impact of the MEL precursor TRP on production patterns was assessed. In parallel, glucose concentrations and oxygen availability were also varied. Two concentrations of glucose (20 and 200 g/L) and TRP (0.17 and 0.87 g/L) were tested. Oxygen levels in the experiments were altered by increasing or decreasing the headspace in fermentation vessels in the presence and absence of agitation. Two *Kazachstania* strains (CBS 9918 and 4311) able to produce MEL together with a *S. cerevisiae* QA23 strain previously reported to produce significant MEL concentrations (Rodriguez-Naranjo et al., 2012) were selected for further detailed investigation. Although not reproducible, MEL was detected only in the cultures grown under oxygen limited conditions without agitation, while neither glucose concentration nor TRP levels affected MEL production by *S. cerevisiae* QA23. The initial data suggested that lower glucose levels but not TRP concentrations increased MEL production by *Kazachstania* species (Table 3.6). However, follow-up experiments that included two additional *Kazachstania* strains (CBS 10706 and CBS 6904) again yielded highly divergent data sets and the high levels of MEL observed in *Kazachstani*a spp cultures observed in Tables 3.5 and 3.6 could not be reproduced (Table 3.7).

Table 3.6: Concentrations of MEL produced by yeast in SGJ medium (50 mL in 100 mL spice jar) without agitation under oxygen limited conditions with different concentrations of glucose and TRP (mean ± range of duplicate samples). Samples were taken after 48 h growth.

Species	MEL concentration in µg/L			
	200 g/L glucose		20 g/L glucose	
	0.87 g/L TRP	0.17 g/L TRP	0.87 g/L TRP	0.17 g/L TRP
S. cerevisiae QA23	0.20 ± 0.01	0.16 ± 0.01	0.18 ± 0.02	0.21 ± 0.02
K.aerobia CBS 9918	199.9 ± 0.9	210.2 ± 1.4	404.0 ± 6.2	406.2 ± 9.6
K.servazii CBS 4311	177.1 ± 3.5	199.9 ± 1.3	335.2 ± 3.4	376.2 ± 16.8

Table 3.7: Concentrations of MEL (duplicate biological determinations) produced by *Kazachstania* spp in SGJ medium with 179 mg/L TRP (50 mL in 100 mL spice jar) under oxygen limited conditions without agitation. Samples were taken after 48 h growth

Species	MEL concentration µg/L	
	Biological 1	Biological 2
K. hellinica (CBS 10706)	0.11	0.06
K. aerobia (CBS 9918)	0.65	2.34
K. servazii (CBS 4311)	0.20	1.35
*K. servazii (*CBS 4311)	0.54	0.37
K. solicola (CBS 6904)	0.70	0.07

3.4.4 Impact of metabolites and co-factors involved in the melatonin biosynthetic pathway, carbon sources and environmental stressors on melatonin production patterns

In order to standardise the experimental conditions and achieve better reproducibility of MEL measurements, experiments were performed in MM (20 g/L glucose) with two different concentrations of TRP (20 mg/L and 100 mg/L). The data thus far, although not reproducible, had suggested that the highest levels of MEL had always been measured within the first 48 h after inoculation. For this reason, more samples were taken during the early culture period to establish a coherent production pattern, and to assess whether the data might reflect an oscillatory character of MEL production. These experiments focused on *S. cerevisiae* IWBT Y805 since this strain produced the highest extracellular levels of MEL in SGJ fermentations in comparison to other *S. cerevisiae* strains (Table 3.5).

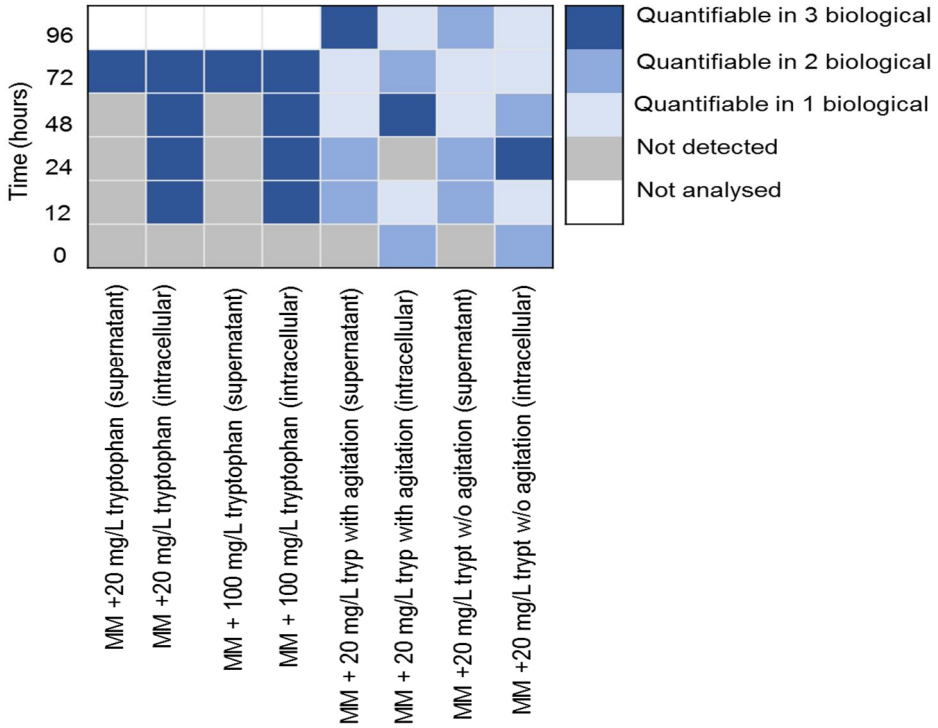

Figure 3.1: Evaluation of extracellular and intracellular production of MEL by *S. cerevisiae* IWBT Y805 in MM with 20 and 100 mg/L TRP with and without agitation.

MEL was not detected in the supernatant for the first 48 h after inoculation but only after 72 h with poor reproducibility (Fig 3.1). Levels of MEL detected at this time point were higher in MM with 100 mg/L TRP (0.22, 0.27 and 0.33 ng/10^7 cells per replicate) when compared to the 20 mg/L TRP (0.1 and 0.17 ng/10^7 cells) treatment where melatonin was detected in two of the three biological replicates. This supports the observations made by Vigentini et al. (2015) where MEL was only quantified in MM supplemented with 100 mg/L TRP. The average intracellular levels of MEL decrease over time from 1.87 ng/10^7 cells at 12 h to 0.05 ng/10^7 cells at 72 h. Higher concentrations of MEL were found in MM with 20 mg/L TRP at 12 h (1.96, 1.44 and 2.19 ng/10^7 cells per replicate) in comparison to MM with 100 mg/L (1.95, 0.94 and 0.97 ng/10^7 cells per replicate) but thereafter slightly higher intracellular concentrations were produced in MM with 100 mg/L TRP, however attempts to confirm this production pattern in subsequent experiments were unsuccessful. MEL production patterns in MM with 20 mg/L TRP under oxygen limited conditions, with and without agitation were also investigated. When detected, the levels of MEL in the agitated cultures were similar to the static cultures in both the extracellular (0.005 ng/10^7 cells in static vs 0.008 ng/10^7 cells in agitated cultures at 12 h) and intracellular samples (0.013 ng/10^7 cells in static vs 0.006 ng/10^7 cells in agitated cultures at 12 h). The highest extracellular concentration was detected in the agitated cultures at 96 h (0.52, 0.31 and 0.48 ng/10^7 cells per replicate). At the same timepoint in the static cultures, melatonin was detected in two of the three biological replicates with lower

concentrations in comparison with the agitated cultures (0.137 and 0.138 ng/10^7 cells per replicate). The highest levels of intracellular MEL were detected immediately after inoculation (0.77 and 0.41 ng/10^7 cells for static and agitated cultures respectively) which was once again quantified in two of three biological repeats. Concentrations decreased rapidly over time with continued inconsistent production patterns.

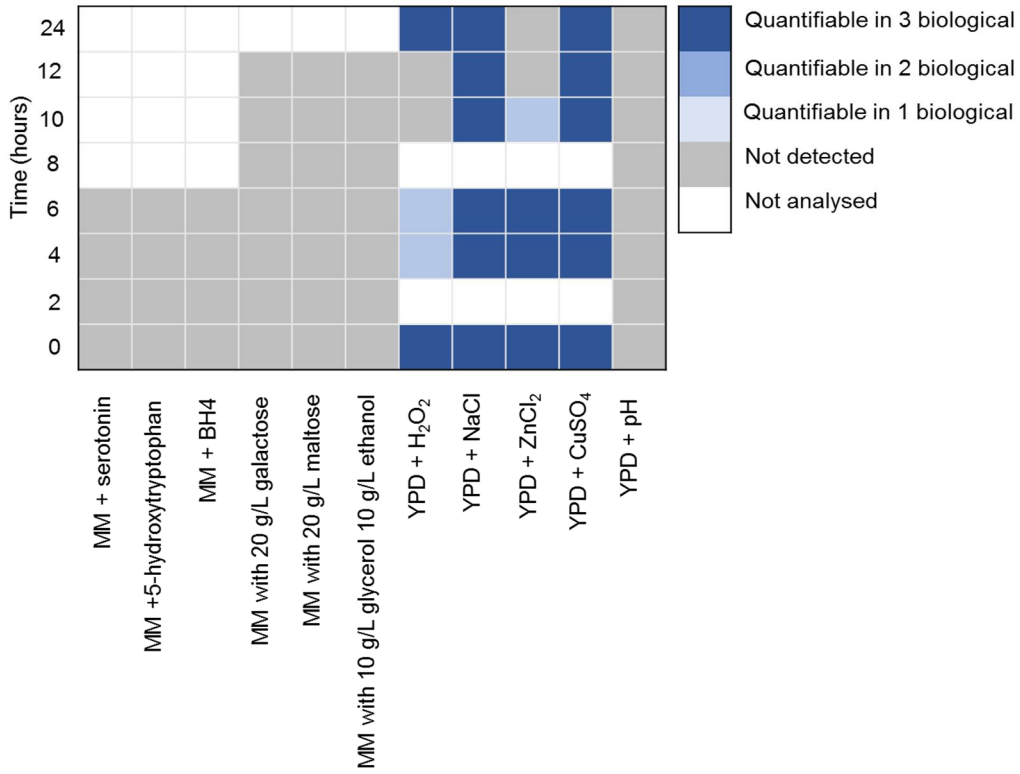

Figure 3.2: Evaluation of extracellular levels of MEL produced by *S. cerevisiae* IWBT Y805 in MM with 1 mM 5-HT and 5-HTP, 50 µM BH4, 20 g/L galactose and maltose respectively, 10 g/L glycerol and ethanol respectively; in YPD with 3.5 mM H_2O_2, 0.4 mM NaCl, 2.5 mM $ZnCl_2$ and 5 mM $CuSO_4$. For the stress experiments in YPD, stressors were added immediately after inoculation.

In a further experiment, *S. cerevisiae* was grown up to 6 h in MM supplemented with 5-HT and 5-HTP as well as BH4 cofactor however MEL was not detected at any time point (Fig 3.2). MEL was not detected in any samples when *S. cerevisiae* was cultured in MM supplemented with 20 g/L glucose, 20 g/L galactose, 20 g/L maltose or 10 g/L glycerol + 10 g/L ethanol as carbon sources.

Studies have shown that MEL production in both plants and animals is stress inducible (Tan et al., 2015). In order to assess if MEL is induced by stress in yeast, *S. cerevisiae* was cultured in YPD supplemented with sublethal concentrations of environmental stressors. None of the stresses led to reproducible MEL production or production pattern. MEL was sometimes detected at very low levels when $ZnCl_2$, H_2O_2, NaCl and $CuSO_4$ were added immediately after inoculation (Fig 3.2). The inoculum free YPD medium was also analysed for melatonin, but

none was detected. There was however no consistency in the production patterns in any of the conditions tested with melatonin detected in only one biological repeat at some timepoints. When NaCl stress was evaluated, the highest MEL level was found at 4 h (0.05, 0.04, 0.082 ppb/OD), thereafter concentrations decreased. On the other hand, MEL concentration as highest at 10 h when the yeast was stressed with $CuSO_4$ (0.008, 0.005 and 0.069 ppb/OD). In the $ZnCl_2$ and H_2O_2 experiments the highest levels of MEL were found immediately after inoculation of cultures into YPD with these stressors and decreased over time. Again, the MEL concentrations produced are highly variable revealing the inconsistency in production by *S. cerevisiae* when stressed. MEL was not detected in the experiments where the stressors were added to cultures in exponential growth phase as well in pH experiments.

3.4.5 Melatonin production in chemostat

3.4.5.1 Melatonin production by *S. cerevisiae* IWBT Y805 under nitrogen limited continuous culture

Data generated thus far suggests that MEL can be produced by *S. cerevisiae*, but that the production patterns were not reproducible in any of the conditions investigated in this study. Some of the data however suggested that TRP levels may have an impact on MEL production (Figure 3.1). Since the cultivation of microorganisms under continuous culture conditions allows for tighter control of nutrient and growth parameters, the impact of TRP on MEL production was carefully monitored in a nitrogen limited chemostat. Samples were taken once cultures reached steady state and ammonia analysis was performed.

Table 3.8. Residual ammonia concentrations (mean ± range of duplicate measurements) under steady conditions in the bioreactor at 0.264/h dilution rate fed with glucose (80 g/L) minimal medium containing various $(NH_4)_2SO_4$ concentrations

	$(NH_4)_2SO_4$ (g/L) added to bioreactor		
	0.3	0.6	5
Ammonia concentrations (mg/L)	0.9 ± 0.03	59.5± 1.6	70 ± 2.1
Maximum specific growth rate (/h) [a]	0.32	0.336	0.335

[a] Maximum specific growth rate determined in batch mode before start of medium pump at a dilution rate of 0.264/h

The data showed that at 0.3 g/L $(NH_4)_2SO_4$ the ammonia concentrations at steady state were close to zero (0.9 mg/L). When the concentration of $(NH_4)_2SO_4$ in feed medium was increased to 0.6 and 5 g/L, the residual ammonia levels at steady state were 59.5 and 70 mg/L respectively (Table 3.8), suggesting that other factors had become limiting. Consequently, 0.3 g/L was selected as the limiting concentration of $(NH_4)_2SO_4$. The maximum growth rate was

of cultures at this concentration of $(NH_4)_2SO_4$ was 0.320 /h (Fig 3.3). Similar conditions were found by Kenyon et al. (1986).

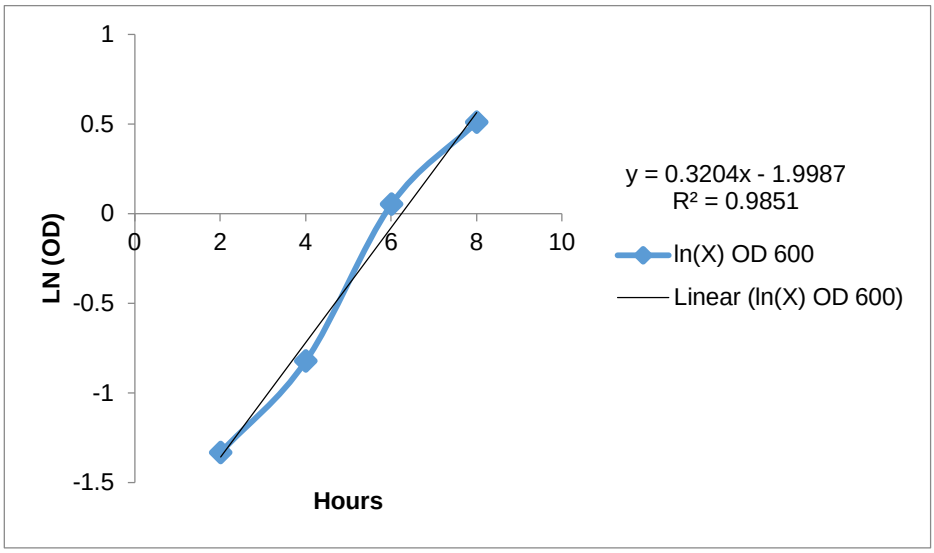

Figure 3.3: Maximum growth rate of *S. cerevisiae* cultured in minimal medium containing 0.3 g/L $(NH_4)_2SO_4$.

Once 50 mg/L TRP was injected into the bioreactor, the levels decreased over time in both intracellular and extracellular samples (Fig 3.4). However, this condition did not result in the production of metabolites (5-HTP, 5-HT, 5-MT, MEL, 6-OHM, 3-IAA) in the MEL biosynthetic pathway; IEA was not measured in these experiments.

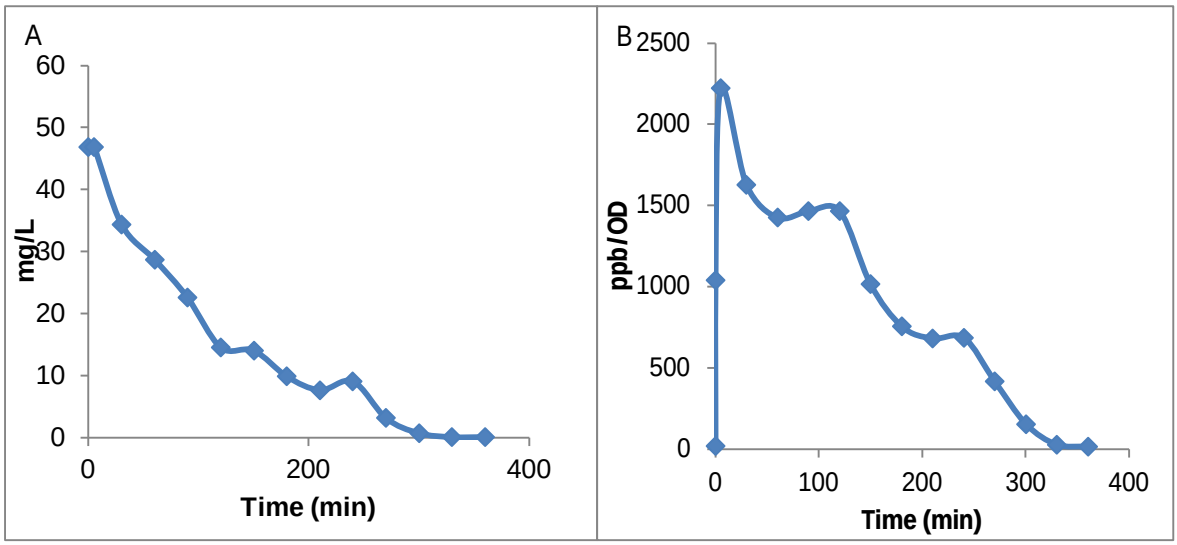

Figure 3.4: Extracellular (A) and intracellular (B) concentrations of TRP in MM (80 g/l glucose) with 0.3 g/L $(NH_4)_2SO_4$ in continuous culture.

3.4.5.2 Melatonin production under tryptophan limited continuous culture conditions

MEL is synthesised from TRP and the batch culture data, though highly variable, showed that supplementation of the medium with this amino acid may have an impact on the MEL levels. In this experiment *S. cerevisiae* IWBT Y805 was cultured in MM containing 80 g/L glucose with 5 mM (1.02 g/L TRP; 140 mg/L N) TRP as a sole nitrogen source to assess if the production levels and consistency of MEL could be improved. As TRP is not a preferred nitrogen source by yeast, the specific growth rate (0.054/h; Fig 3.5) was considerably lower than when ammonium sulphate was the limiting nitrogen source (0.32/h; Fig 3.3).

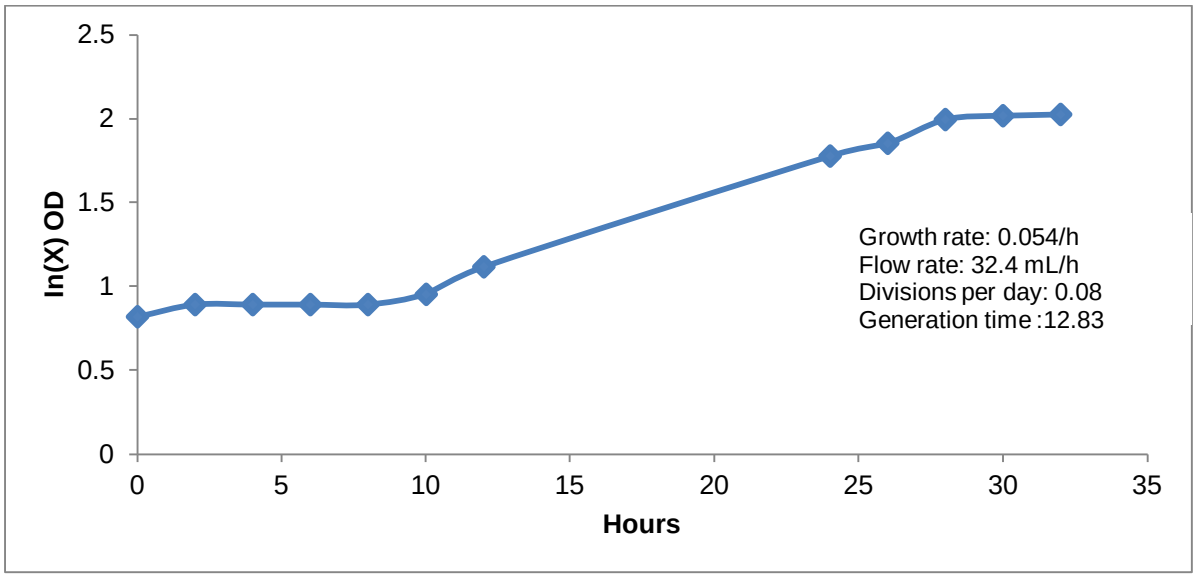

Figure 3.5: Growth of *S. cerevisiae* in MM with 80 g/L glucose and 5 mM (1.02 g/L) TRP as sole nitrogen source

At steady state, the amount of TRP measured in the supernatant was approximately 0.2 g/L indicating that the yeast cells utilised 0.8 g/L TRP. 5-HTP and 5-HT, but not MEL, were detected in both the intracellular and extracellular samples. Intracellular concentrations of 5-HT were lower than the other metabolites. (Table 3.9).

Table 3.9: Steady state concentrations of TRP, 5-HTP and 5-HT in the intra- and extracellular samples (mean ± range of duplicate measurements) of *S. cerevisiae* IWBT Y805 cultured in MM with TRP as sole nitrogen source. Samples were taken over 6 h in one day.

	TRP		5-HTP		5-HT	
Sample	Intracellular (µg/L)	Extracellular (g/L)	Intracellular (µg/L)	Extracellular (µg/L)	Intracellular (µg/L)	Extracellular (µg/L)
1 (09:00)	615.8 ± 55.6	0.190 ± 0.0002	151.75 ± 0.1	153.1[a]	0.01[a]	0.90 ± 0.33
2 (12:00)	1418.4 ± 128.3	0.182 ± 0.011	153.2 ± 0.1	154 ± 0.85	0.58 ± 0.04	1.72 ± 0.3
3 (15:00)	1616.8 ± 159	0.164 ± 0.006	155.4 ± 0.05	152 ± 2.7	0.1 ± 0.02	0.97 ± 0.4

[a] Quantified in only one of the biological samples.

In a subsequent repeat of this experiment, neither 5-HT, 5-HTP nor any of the other metabolites in the MEL biosynthetic pathway were detected. However, other metabolites synthesised from TRP were present (Fig 3.6). In both the intracellular (26.4 – 46.7 mg/L) and extracellular (31 – 33 mg/L) samples, IEA was the most abundant metabolite across all time points. However, a significant portion of TRP metabolized was probably catabolized as the nitrogen source for growth and biomass formation. Concentrations of 3-IAA quantified were much lower than IEA. When the distribution of 3-IAA between the cells and cell-free medium volume were calculated, the intracellular concentrations were higher than the extracellular concentrations (304.8 – 886.1 µg/L vs 51 – 72.6 µg/L respectively).

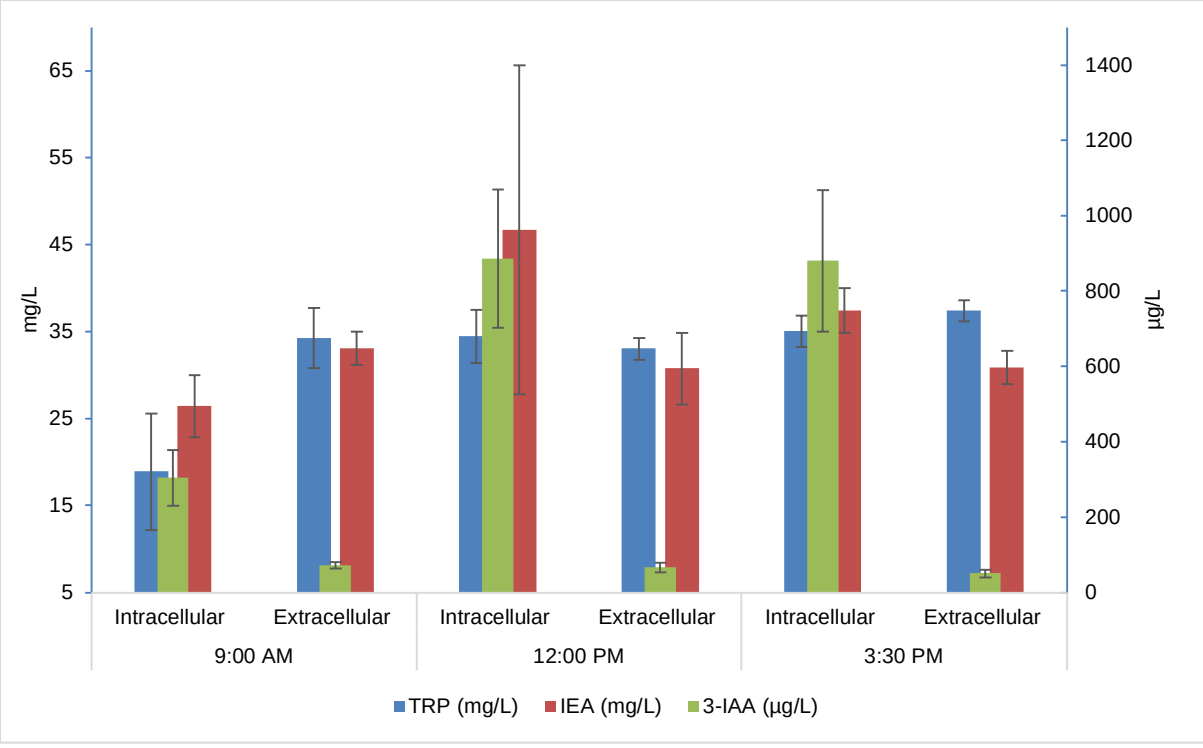

Figure 3.6: Concentrations of TRP, IEA and 3-IAA in extracellular and intracellular samples (mean ± range of duplicate measurements) of *S. cerevisiae* IWBT Y805 in continuous culture with TRP as sole nitrogen source measured over 6 h.

3.4.5.3 Influence of light/dark cycle on the synthesis of metabolites in continuous culture conditions

Using measurements of glyceraldehyde-3-phosphate as an indicator of circadian day length, Dick et al. (2013) showed that *S. cerevisiae* had circadian day lengths of 24 h, 25 h and 40 h. As MEL production has been shown in animals to be related to day light/dark cycles (Reiter, 1991; Tan et al., 2015), the potential impact of an artificial light cycle on the synthesis of metabolites in the MEL biosynthetic pathway was investigated. Cultures were grown in MM containing 80 g/L glucose supplemented with 100 mg/L TRP and 0.2 g/L $(NH_4)_2SO_4$. The specific growth rate at this concentration of $(NH_4)_2SO_4$ was 0.319/h (Fig 3.7). However, a dilution rate 0.307/h was set to prevent washout of cultures.

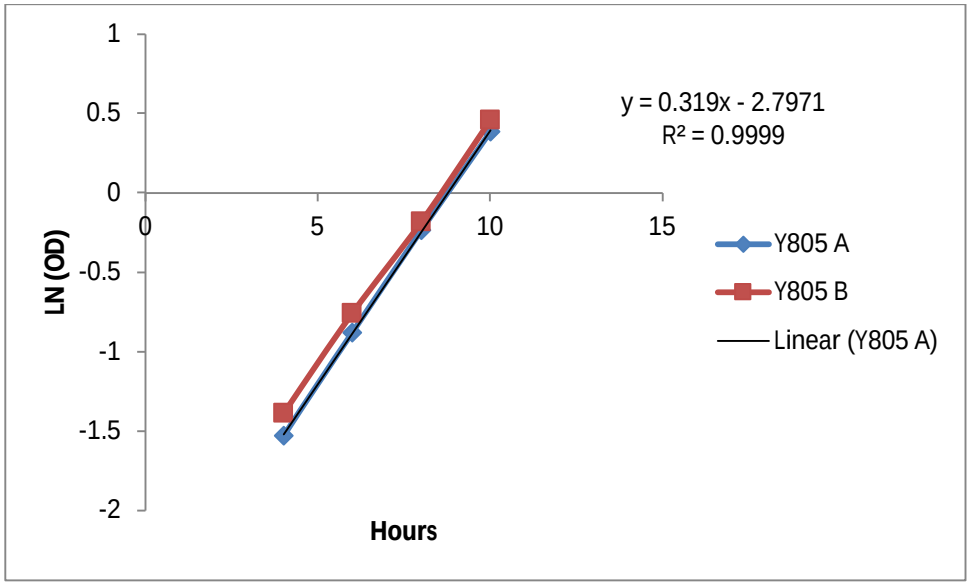

Figure 3.7: Growth rate in exponential growth phase of *S. cerevisiae* cultured in MM containing 80 g/L glucose with 100 mg/L TRP and 0.2 g/L $(NH_4)_2SO_4$.

The level of ammonia at steady state were 0.33 mg/L and increased to above 2 mg/L at the end of the experiment whereas the glucose levels remained relatively stable above 70 g/L (Table 3.10). OD measurements remained stable (OD 2.81 – 2.84) over the course of the experiment.

Table 3.4. Residual ammonia and glucose concentrations (mean ± range of duplicate measurements) under steady conditions in the bioreactor at 0.307/h dilution rate fed with MM containing glucose (80 g/L), 0.2 g/L $(NH_4)_2SO_4$ and 100 mg/L TRP

	Sampling time points in light/dark cycle			
	4 am (dark)	7 am (dark)	4 pm (light)	7 pm (light)
Ammonia (mg/L)	0.33 ± 0.17	1.16 ± 0.6	2.09 ± 0.70	2.85 ± 0.6
Glucose (g/L)	74.71 ± 2.03	70.61 ± 4.35	72.92 ± 1.76	72.71 ± 1.25

Metabolites in the MEL pathway were not found in any of the samples. Intracellular concentrations of TRP were consistently higher than the extracellular concentrations of this amino acid. As observed in the previous experiment, the concentrations of IEA observed in both the supernatant and intracellular samples were higher than concentrations of 3-IAA. An interesting 12 h cyclical pattern in intracellular IEA is observed with higher concentrations at the 7am and 7pm time points and lower concentrations at the 4am and 4pm timepoints but these variations did not coincide the periods of light and dark. 3-IAA was quantified in the supernatant samples across all timepoints with higher concentrations measured in the initial two sampling periods. No intracellular 3-IAA was detected. (Table 3.11).

Table 3.11: Concentration of TRP, IEA and 3-indoleacetic acid (mean ± range of duplicate samples) in extracellular and intracellular samples of *S. cerevisiae* IWBT Y805 cultured in nitrogen limited continuous culture conditions. Steady state cultures were exposed to an artificial 12 h day/night cycle for 3 days before samples were taken.

	4:00 AM (dark)		7:00 AM (dark)		4:00 PM light)		7:00 PM (light)	
	Intracellular	Extracellular	Intracellular	Extracellular	Intracellular	Extracellular	Intracellular	Extracellular
TRP (mg/L)	2.03 ± 0.32	1.24 ± 0.15	5.28 ± 0.25	0.95 ± 0.50	3.02 ± 0.29	0.77 ± 0.31	7.95 ± 3.03	0.50 ± 0.19
IEA (mg/L)	1.04 ± 0.17	0.28 ± 0.02	2.03 ± 1.25	0.35 ± 0.02	0.84 ± 0.12	0.19 ± 0.003	3.88 ± 0.35	0.11 ± 0.01
3-IAA (µg/L)	nd[a]	8.5 ± 2.5	nd	8.8 ± 0.9	nd	4.4 ± 0.6	nd	3 ± 0.4

[a]Not detected

3.4.6 *In Silico* analysis to identify genes potentially involved in biosynthetic pathway in yeast

The biosynthetic pathway of MEL in animals and plants has been characterised but remains unclear in microorganisms. Tan et al (2016) hypothesised that the biosynthetic pathway in *S. cerevisiae* similar to the animal pathway differing only with the penultimate metabolite and the ultimate enzyme involved in the reaction. The authors described the substrate affinity of yeast AANAT to 5-HT and 5-methoxytryptamine. Yeast AANAT has a higher substrate affinity for 5-MT than 5-HT making 5-MT the rate limiting metabolite and AANAT the ultimate enzyme in

the synthetic pathway in yeast (Fig 3.8). Using the sequences of proteins involved in the synthesis of each intermediate in the biosynthetic pathway in both animal and plants as well as conserved regions of these proteins, potential orthologs in yeast were searched for.

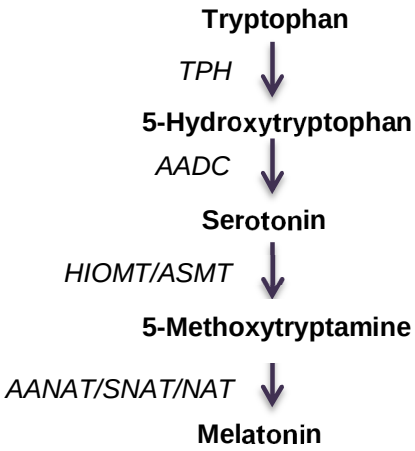

Figure 3.8: Hypothetical melatonin pathway in yeast. TPH, tryptophan hydroxylase; AADC, aromatic amino acid decarboxylase; ASMT, *N*-acetylserotonin methyltransferase; AANAT, arylalkylamine N-acetyltransferase; SNAT, serotonin *N*-acetyltransferase (Sprenger et al. 1999; Tan et al. 2016).

The blasted protein sequences of these enzymes were aligned on EggNOG$_{4.5.1}$ (Huerta-Cepas et al., 2016) and OrthoDB v10 (Kriventseva et al., 2019) phylogenetic platforms to identify the organisms they occurred in as well as orthologous sequences in other classes of organisms. Using bioinformatic platforms the conserved domain in each enzyme group were identified. Orthologous sequences of TPH were found in several species in the animal kingdom but not in plants, bacteria nor yeast. TDC and AADC have a common conserved domain, however their enzymatic activity seems to be restricted to plants and animals respectively. Orthologs for the plant T5H were found only in bacteria whereas ASMT was found in plants and animals. Orthologs for AANAT sequence was found in all organisms (Table 3.12).

Table 3.12: Enzymes involved in the biosynthesis of MEL in plants and animals and the presence in different classes of organisms.

	TPH	TDC	T5H	AADC	ASMT	AANAT/SNAT
Number of species in phylogenetic alignment	105	976	1076	1015	821	620
Conserved Domain	Biopterin dependent hydroxylase	pyridoxal-dependent decarboxylases	Cytochrome P450	pyridoxal-dependent decarboxylases	Methyltransferases	Acetyltransferase
Vertebrates	Present	Present	Absent	Present	Present	Present
Invertebrates	Present	Present	Absent	Present	Present	Present
Plants	Absent	Present	Present	Present	Present	Present
Bacteria	Absent	Present	Present	Present	Absent	Present
Yeast	Absent	Absent	Absent	Absent	Absent	Present

TPH – tryptophan hydroxylase; TDC – tryptophan decarboxylase; T5H – tryptamine 5-hydroxylase; AADC – aromatic L-amino acid decarboxylase; ASMT – *N*-acetylserotonin methyltransferase; AANAT - arylalkylamine N-acetyltransferase; SNAT - serotonin N-acetyltransferase.

The conserved domains of each enzyme were used to generate consensus patterns on PROSITE. The consensus pattern of the conserved region in the central part of tryptophan hydroxylase contains two conserved histidines that are involved in the binding to iron. No matches were found when this pattern was cross- referenced on *Saccharomyces* genome database. Decarboxylases in animals and plants generated the same pattern on PROSITE and when cross referenced with yeast genome, no matches were found. The same observation was made with the pattern generated from the T5H protein sequence. The N-acetyltransferase and methyltransferase protein generated MATRIX sequences and cross referencing of the SAM-dependent O-methyltransferase class II-type matrix found no matches in yeast (Table 3.13). Several N-acetyltransferase were found in yeast database but the only enzyme that had aralkylamine N-acetyltransferase activity was *PAA1*. However, studies have shown that it is not involved in MEL synthesis in yeast (Ganguly et al., 2001).

Table 3.13: Consensus patterns of conserved regions in the protein sequences of enzymes involved in MEL biosynthesis. Patterns were generated on PROSITE.

Protein	PROSITE entry	Description	Pattern
Tryptophan hydroxylase	PS00367	Biopterin-dependent aromatic amino acid hydroxylases signature	P-D-x(2)-H-[DE]-[LIVF]-[LIVMFY]-G-H-[LIVMC]-[PA]
Decarboxylase	PS00392	Pyridoxal-phosphate attachment site	S-[LIVMFYW]-x-{KG}-x(3)-K-[LIVMFYWGH]-[LIVMFYWG]-x-{R}-x-[LIVMFYW]-{V}-[CA]-x(2)-[LIVMFYWQ]-{K}-x-[RK]
Tryptamine 5-hydroxylase	PS00086	Cytochrome P450 cysteine heme-iron ligand signature.	[FW]-[SGNH]-x-[GD]-{F}-[RKHPT]-{P}-C-[LIVMFAP]-[GAD]
N-acetyltransferase	PS51186	Gcn5-related N-acetyltransferase (GNAT) domain profile	MATRIX
Methytransferase	PS51683	SAM-dependent O-methyltransferase class II-type profile	MATRIX

3.5 Discussion

This study has shown that production of melatonin by yeast is inconsistent and conditions that resulted in reproducible melatonin production were not identified. Numerous studies have investigated the synthesis patterns of MEL in different organisms and the oscillatory pattern of MEL production in animals and plants seems to be related to day and night cycles, with peak levels reached at night in animals and during the day in plants (Pevet and Challet, 2011; Tan et al., 2012; Reiter et al., 2015). Endogenous oscillations and rhythms of living organisms are based of feedback loops in complex biochemical reaction networks. For example, in some circadian rhythms light plays an important role in providing energy that is directly or indirectly involved in the oscillating phenomena (Luttge and Beck, 1992). In yeast, however this synthesis pattern is not well understood and did not respond to circadian patterns in the experiments conducted in this study. Several studies have documented oscillations of metabolites in yeast which include glycolytic (frequency of 0.5 min^{-1} to 4.6 min^{-1}) and respiratory (frequency of 1-40 h) oscillations (Richard, 2003). In continuous culture conditions, studies found that *S. cerevisiae* had sustained oscillations of metabolites (Hess and Boiteux, 1971). However, sampling periods might not have been appropriate to detect consistent oscillation in MEL production since the experimental set-up only allowed sampling over extended time periods. To assess the potential short- and long-term oscillatory patterns of MEL in yeast, frequent samples taken every minute over a 24 h period would be needed.

Rapid, real-time analysis using single cell mass spectrometry could overcome the practical limitations associated with frequent sampling. By using microprobe extraction or laser desorption/ionization, single cell mass spectrometry techniques have analysed cellular contents of mammalian and plant cells in real-time (Standke et al., 2019). This technology could be used to shed more light on MEL production in yeast at single cell level which might have been masked by the bulk sampling employed in this study as well as others.

The influence of environmental stressors, supplementation of MEL pathway intermediates, nitrogen source as well as an artificial circadian rhythm on MEL production in intracellular and extracellular samples was assessed in this study. Although MEL was detected in the different growth media used, there was consistent inter- and intra-experimental variability in the concentrations of the indoleamine quantified and an absence of oscillatory production patterns. The inconsistencies in quantification of MEL in these experiments may also be due to rapid conversion of this indoleamine to other metabolites. Poeggeler and Hardeland (1994), Tan et al. (2002), 2015) reported on the rapid degradation of MEL and other 5-methoxylated related indoles due to photooxidation, hydroxylation by free radicals or enzyme metabolization. Where possible, extreme care was taken to protect experimental set-ups and samples from exposure to light in this study. Some studies have shown that the rhythmicity of oscillations could be disrupted by external stimuli such as a change in light intensity (Buchanan-Bollig, 1984; Luttge and Beck, 1992). When studying the circadian rhythm of CO_2 exchange in the crassulacean-acid-metabolism plant *Kalanchoë daigremontiana*, Buchanan-Bollig (1984) showed that long-lasting endogenous oscillations built up as the intensity of the continuous illumination gradually increased from darkness. However, these oscillations were inconsistent when the irradiance became higher. Although extreme care was taken to ensure that biological samples were handled in the same way, minute differences in culture conditions could have resulted in the inconsistent melatonin production patterns observed in this study as well as others (Fernández-Cruz et al., 2018, 2019).

Earlier studies on MEL used immunoassays and GC-MS which had limitations that resulted in an over- or underestimation of MEL content in a sample (Garcia-Parrilla et al., 2009). Several studies found that LC-MS/MS was sensitive enough to accurately detect and quantify low levels of MEL and other TRP metabolites in different matrices (Eriksson et al., 2003; Cao et al., 2006; Vigentini et al., 2015; Fernández-Cruz et al., 2016). The sensitivity of this method to measure MEL in samples was confirmed with the limit of detection being 0.01 ng/mL. However, conditions that resulted in consistent MEL production were not identified, confirming the inconsistencies observed in other studies. When Fernández-Cruz et al (2017) followed the production patterns of indolic compounds during fermentation in synthetic must, they found irregular occurrences of melatonin as it tended to appear and disappear in the

medium as time progressed and concentrations ranged from 0.4 ng/mL to 2.24 ng/mL. In natural grape must fermentations these authors observed in their time plots revealed zigzag trends in MEL production and more than one peak in the concentration of this indoleamine in Corredera and Moscatel cultivars (Fernández-Cruz et al., 2018). Vigentini et al., (2015) quantified MEL mainly in the first 48 h in cultures grown in MM with 100 mg/L tryptophan. In this study the concentrations of MEL produced were dependent on yeast strain used more than the cultivation medium (Tables 3.4 and 3.5). A recent study reported on the intracellular production of MEL by various non-*Saccharomyces* yeast strains during fermentation; however concentrations reported were very low (reported in ng/10^8 cells) with some variations in the production patterns observed (Fernandez-Cruz et al., 2019). In the instances where MEL was quantifiable in this study, it was at lower biomass levels (10^7 cells) but higher concentrations of this indoleamine (µg/L) were quantified in select instances. Therefore, increasing biomass of cells used to extract and quantify MEL could improve the consistency of levels detected in experiments but that may not result in higher detectable concentrations as was observed in the Fernandez-Cruz (2019) study.

The synthesis pathway of MEL from TRP is not established yet in yeast. However, it is hypothesised that more than one pathway could be involved in animals and plants respectively. These pathways involved several metabolites including 5-HTP, tryptamine, 5-HT, N-acetylserotonin, 5-MT and MEL. Studies have shown that MEL production can be induced by environmental factors and this was explored from different angles here. The first angle explored was the influence of biosynthetic pathway intermediates on MEL production. A few studies have assessed the influence of enzymes and metabolites in biosynthetic pathway on the levels of MEL produced by yeast. Sprenger et al (1999) found that supplementation of a medium with 5-HT or N-acetylserotonin resulted in an increase in MEL production by *S. cerevisiae*. Supplementation of the medium with 5-HTP and 5-HT did not result in the production of MEL in this study. A possible reason for this is that 5-HT may not be the rate limiting substrate in MEL production in yeast. In the classic MEL biosynthetic pathway in animals, AANAT converts 5-HT to N-acetylserotonin or alternatively in the hypothesised predominant pathway, AANAT converts 5-MT to MEL (Tan et al., 2016). As mentioned previously, yeast AANAT has a higher substrate specificity for 5-MT than 5-HT (Ganguly et al., 2001). Muñiz-Calvo (2019) found that addition of 5-MT to the medium could stimulate consistent production of MEL by yeast. These authors also found that 5-HTP supplementation mainly resulted in the synthesis of TRP and to a lesser extent tryptamine, 5-HT and N-acetylserotonin. However, bottlenecks in the system still exist as supplementation of a metabolite did not lead to the synthesis of all downstream metabolites. In some cases, the opposite was observed where 5-HT supplementation resulted in the production of 5-HTP.

Germann et al (2016) constructed a MEL producing yeast by inserting tryptophan hydroxylase (TPH) genes from *Schistosoma mansoni* or *Homo sapiens*, *Homo sapiens* 5-hydroxy-L-tryptophan decarboxylase (*HsDDC*), *Bos Taurus BtAANAT* and *Homo sapiens HsASMT*. They highlighted the importance of the co-factor BH4 in the functioning of TPH and overexpressed *Rattus norvegicus* 6-pyruvoyl-tetrahydropterin and sepiapterin reductase; 4a-hydroxytetrahydrobiopterin dehydratase from *Lactobacillus ruminis* or *Pseudomonas aeruginosa* and 6-pyruvovyl-tetrahydropterin synthase from *Homo sapiens* or *Rattus norvegicus* in *S. cerevisiae*. These genes are involved in the biosynthesis and regeneration pathways of BH4 which is not present in yeast. (Germann et al., 2016). Supplementation of chemically synthesised BH4 in this study did not result in the production of MEL but BH4 has a very short half-life of approximately 16 min and completely disappears from saline in 90 min (Kaufman, 1967; Stone, 1976). Sampling points could have inadvertently missed the point at which any of the pathway intermediates were produced. *In silico* analysis was unsuccessful in identifying orthologs of the plant and animal enzymes involved in the biosynthetic pathway, with the exception of *PAA1* which has been reported previously (Ganguly et al., 2001). Therefore, the pathway used by yeast to synthesize MEL remains unclear. Enzyme cooperativity plays a primary role on the occurrence of periodic behaviour (Cohen and Goldbeter, 2004). The apparent absence of dedicated melatonin biosynthesis enzymes in yeast as well as the substrate promiscuity of some of the enzyme families producing metabolites related to melatonin could be contributing factors to the inconsistency in production patterns observed. An example of the promiscuity is found in AANAT family of enzymes. These enzymes acetylates arylalkylamines which include serotonin, tryptamine, and phenylethylamine but, the fungal homolog of this enzyme also acetylates polyamines at the same rate as arylalkylamines and has therefore also referred to as a polyamine acetyltransferase (Ganguly et al., 2001; Klein, 2007). The diversity of enzymes in this pathway is not limited to substrate specificity as ASMT in plants also has at three isoforms (Tan et al., 2016). These observations suggest that there is a possibility that other enzyme systems may be involved in the production of MEL in yeast, but this needs further investigations.

MEL is also possibly produced as an overflow metabolite in conditions that are yet to be identified. As mentioned above, abiotic environmental stressors such as salt, chemical and temperature can stimulate the production of MEL in plants and animals (Tan et al., 2010; Li et al., 2016). Most of the yeast strains found to produce detectable levels of MEL in this study (Table 3.1) were isolated from grape must collected in the Stellenbosch region of South Africa. These cultures may have produced MEL in response to the varying environmental conditions in the vineyards. The results of the stress experiments although inconsistent suggest that yeast synthesise MEL as a defence mechanism. Varying concentrations of MEL were

produced when *S. cerevisiae* was exposed to oxidative, heavy metal and salt stress but further experiments are required to improve the characterisation of environmental stressor on MEL production in yeast. Contrary to previous reports (Rodriguez-Naranjo et al., 2012; Vigentini et al., 2015), the exponential growth phase did not consistently correlate with highest level of melatonin in this study. In MM with 20 mg/L tryptophan (intracellular samples) (Fig 3.1) as well as the $ZnCl_2$ and H_2O_2 stress experiments in YPD (Fig 3.2), highest levels of melatonin were found immediately after inoculation when cultures were still in lag phase. The highest MEL concentrations in the extracellular MM was found when cultures were in stationary phase. The initial batch experiments found that TRP may have an impact on the levels of MEL produced by yeast and this was explored further in a TRP limited continuous culture. Although MEL was not detected in any of these experiments, 5-HTP and 5-HT were quantified in conditions were TRP was used as a sole nitrogen source. This further supports the hypothesis that one of the major biosynthetic pathways in yeast involve the conversion of TRP to 5-HTP by TPH and then AADC decarboxylates 5-HTP to 5-HT. Further studies are needed to confirm the underlying enzymatic reactions in the biochemical pathway followed. A recent publication by Muñiz-Calvo et al (2019) hypothesized that the main biosynthetic pathway in yeast involves the conversion of TRP to tryptamine as the highest amount of serotonin quantified in their study was observed after tryptamine supplementation. In this pathway, tryptamine is hydroxylated to 5-HT which can be then converted to *N*-acetylserotonin and 5-MT. The latter metabolite is thought to be the preferred substrate to produce MEL. However, both pathways could be involved in the synthesis of MEL in yeast. IEA and 3-indoleacetic acid were the most abundant metabolites quantified in the TRP experiments.

Even though the mechanisms involved in production of MEL by yeast remains unclear, the wide array of conditions tested in this study shed more light on this topic. The data from these experiments show that MEL production by yeast is not always linked to exponential growth phase as this metabolite was mainly produced intracellularly in the lag phase and released into the medium in the stationary phase. However, it was also shown that yeast produce MEL in response to abiotic stress. By using TRP as a sole nitrogen source, it was shown experimentally that the first two intermediates in the animal and plant biosynthetic pathway of 5-HTP and 5-HT are present. Further studies are required to investigate the combination of environmental parameters, TRP concentrations and supplementation of other pathway intermediates in order to optimise conditions in which this indoleamine is produced in yeast. However, melatonin seems to be an excess metabolite in yeast as it cannot be consistently stimulated in response to environmental changes.

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Chapter 4

Melatonin modulates the transcriptional response of *S. cerevisiae* to hydrogen peroxide oxidative stress

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Melatonin modulates the transcriptional response of *S. cerevisiae* to hydrogen peroxide oxidative stress

4.1 Abstract

Melatonin is a ubiquitous indoleamine and its antioxidant activities are well researched and documented in different biological kingdoms. Melatonin has previously been shown to alleviate oxidative stress in yeast, but the underlying molecular mechanisms remain unclear. This study investigated the transcriptional response of *Saccharomyces cerevisiae* to exogenous melatonin treatment pre- and post-hydrogen peroxide (H_2O_2) stress. Experiments were performed in growth rate-controlled nitrogen-limited continuous culture conditions. Optical density measurements showed that melatonin treated cells recovered quicker than the untreated cells after the addition of H_2O_2. Analysis of RNAseq data revealed that melatonin modulated the expression of a number of genes responding to H_2O_2 stress compared to melatonin untreated cultures. Prior to the induction of stress, melatonin enhanced the expression of several genes in the sulphate assimilation pathway genes which leads to the formation of cysteine and methionine. These amino acids are involved in the formation of glutathione. After stress induction, melatonin enhanced the upregulation of glutathione related oxidative stress response genes (*GLR1, GPX2* and *GRX6*) as well as the transcription factors *MSN2/4*. The data also showed that melatonin influenced several genes in the ergosterol biosynthetic pathway genes. In summary the data revealed the influence of melatonin on expression patterns of transcription factors, glutathione and glutaredoxin pathway enzymes and describes for the first time its influence on the sulphate assimilation and steroid biosynthesis pathways in yeast.

Key words: Transcriptome, *Saccharomyces cerevisiae*, melatonin, oxidative stress

4.2 Introduction

Melatonin is a multifunctional indoleamine found in many living organisms. Since its discovery in animal and plants, numerous studies have characterised its biosynthetic pathways and biological functions in these kingdoms. The known biological functions of melatonin in vertebrates include regulation of circadian rhythms and antioxidant properties but additional impacts on immunomodulation, oncostasis and aging have been suggested. (Tan et al., 2010; Fernández-Mar et al., 2012). The antioxidant capacity of this indoleamine is increased by its secondary and tertiary metabolites which neutralise toxic oxygen derivatives (Tan et al., 2015).

In comparison to vitamin C, vitamin E, glutathione and NADH, the antioxidant capacity of melatonin was shown to be superior in *in vitro* and *in vivo* studies on vertebrates (Gitto et al., 2001; Tan et al., 2003). In addition to its ability to directly scavenge free radicals, melatonin increases messenger RNA and protein levels of major antioxidant enzymes thereby stimulating antioxidant defences (Mayo et al., 2002). It was also shown to protect proteins, lipids and DNA from damage caused by free radicals (Fernández-Mar et al., 2012). Several studies have investigated the mechanisms behind the anticancer activities of melatonin. In one such study, melatonin treatment down-regulated oncogenic genes and up-regulated tumour suppressor genes in MCF-7 human breast cancer cells (Lee et al., 2013).

Some of the diverse biological functions of this indoleamine described in plants include improvement of seed germination, fruit ripening, photosynthesis, circadian rhythm, biotic and abiotic stress tolerance. Studies investigating the functional role of melatonin in plants have used exogenous melatonin treatments (Sharif et al., 2018) or melatonin rich transgenic plants (Kang et al., 2010) to understand the regulation and expression of genes in different plants under various stress conditions. Melatonin treatment in cabbage samples up-regulated specific transcription factors which regulate the expression of anthocyanin biosynthetic genes resulting in an increase in the total antioxidant capacity (Zhang et al., 2016). Shi et al. (2015) showed that exogenous application of melatonin improved the tolerance of bermudagrass to salt, drought and cold stress. Genome wide transcriptomic profiling of the melatonin pre-treated grass in this study revealed that genes involved in nitrogen metabolism, carbohydrate metabolism, tricarboxylic acid transformation, hormone metabolism, metal handling, redox and secondary metabolism were enriched. Microarray analysis of transgenic rice plants overexpressing sheep serotonin N-acetyltransferase revealed that melatonin up-regulated signalling genes and transcription factors such as leucine rich repeat and zinc finger genes and down-regulated senescence associated genes (Byeon et al., 2013).

Several studies have documented the production of melatonin by *S. cerevisiae* and non-*Saccharomyces* yeast strains. However, the biosynthetic pathway and functions of this indoleamine in these microorganisms remain obscure. The protective role of melatonin against H_2O_2-induced oxidative stress and UV stress in yeast was recently described (Vázquez *et al.*, 2017; Bisquert *et al.*, 2018). In these batch culture studies, melatonin reduced reactive oxygen species (ROS) accumulation, enhanced the expression of endogenous antioxidant genes in H_2O_2 stressed *S. cerevisiae* and increased cell viability after exposure to UV and oxidative stress. However, the exact mechanisms behind the protective effects observed remain unclear. This study aimed to understand the transcriptional response of *S. cerevisiae* IWBT Y805 to exogenous melatonin treatment and the temporal response of melatonin treated cultures to H_2O_2-induced oxidative stress. Contrarily to previous studies, a steady state

continuous culture set-up was used which reduced the changes to the physico-chemical conditions associated with batch culture systems. This constant environmental milieu focused the transcriptional response on melatonin-specific mechanisms. Experiments were conducted in nitrogen-limited continuous culture in order to control the cell growth rate and environmental conditions.

4.3 Materials and methods

4.3.1 Yeast strains and media

S. cerevisiae (IWBT Y805) was obtained from the Institute for Wine Biotechnology culture collection of Stellenbosch University. The yeast strain was maintained at -80°C in 15% (v/v) glycerol and was reactivated by streaking out on yeast peptone dextrose (YPD) agar plates containing 10 g yeast extract, 20 g peptone, 20 g glucose and 20 g bacteriological agar per litre. Cultures were stored at 4°C for short term use.

4.3.2 Characterisation of nitrogen limited growth

Nitrogen requirements were determined in batch culture by varying the concentrations of $(NH_4)_2SO_4$ in minimal medium (80 g/L glucose; 1.7 g/L yeast nitrogen base (YNB) without amino acids and ammonium, pH 4.2 (Difco, Heidelberg, Germany)). The following nitrogen concentrations were used; 0.3, 0.6 and 5 g/L. Strain IWBT Y805 was precultured overnight in YNB with 20 g/L glucose and 5 g/L $(NH_4)_2SO_4$ and inoculated into 600 mL glucose minimal medium to an initial OD_{600} of 0.2 in 1L BioFlo® 110 reactor (New Brunswick Scientific Enfield, CT). Cultures were grown at 25°C with agitation at 300 rpm. Sterile compressed air was sparged into the bioreactor at a constant flow rate of 0.5 vvm with the dissolved oxygen tension maintained above 50% of saturation. Growth was monitored spectrophotometrically. Samples were taken to determine glucose and ammonia concentrations.

4.3.3 Nitrogen-limited continuous culture fermentation

Nitrogen-limited concentrations determined under batch conditions were confirmed in continuous culture. Experiments were performed in 1L BioFlo® 110 bioreactor in minimal medium described above. The dilution rate for each nitrogen concentration was determined by the specific growth rate of the cultures in the minimal liquid medium. This was calculated from OD measurements taken during exponential growth phase in batch culture described above where OD_0 is initial OD and OD_t is the OD at time *t.*

$$D = \ln (OD_t/OD_0)$$

The cultures were grown for three residence times (residence time = 3.7 h) to achieve steady state which was confirmed by stable absorbance readings at 600 nm. Once steady state was reached, samples were collected to determine the concentrations of glucose and ammonia. An $(NH_4)_2SO_4$ concentration of 0.3 g/L was determined to be limiting for *S. cerevisiae* and was used for further continuous culture experiments.

4.3.4 Hydrogen peroxide induced stress in continuous culture conditions

Steady state cultures were stressed with 0.6 and 1 mM H_2O_2 in order to determine a sublethal concentration. H_2O_2 was injected aseptically into the bioreactor and sampling was conducted at 0, 15 min, 30 min, 60 min, and 120 min. Growth was monitored spectrophotometrically at 600 nm. To assess the impact of melatonin on oxidative stress, this indoleamine was injected into the feed reservoir to a final concentration of 300 µM. This concentration was shown to protect *S. cerevisiae* yeast strains against redox and oxidative stressors on plate assays reported in Chapter 5. In addition, a study by Tan *et al.* (2000) found that 300 µM melatonin was able to scavenge H_2O_2. The melatonin containing minimal medium was fed into the bioreactor by a previously calibrated peristaltic pump for one residence time before induction of H_2O_2 stress as described above. The bioreactor, feed reservoir and all tubes were covered in aluminium foil to limit exposure of melatonin to light.

4.3.5 Analytical methods

Cell suspensions were centrifuged at 12000 rpm for five min and supernatants collected. The concentrations of glucose and ammonia were measured using specific enzymatic kits for glucose and ammonia (Thermo Fisher Scientific Oy, Finland) and analysed using Arena 20XT photometric analyser (Thermo Electron Oy, Helsinki, Finland). Tryptophan, 5-hydroxytryptophan, 5-methoxytryptamine, serotonin, melatonin, 3-indole acetic acid, tryptophol and 6-hydroxymelatonin (Sigma-Aldrich, St. Louis, MO, USA) were analysed by UPLC-MS/MS analysis carried out on an Acquity UPLC separation module (Waters, Milford, MA, USA) coupled with a triple quadrupole mass spectrometer mod. Xevo TQ MS (Waters).

4.3.6 RNA-extraction and RNA-sequencing

Total RNA was extracted from two biological repeats as described by Collart and Oliviero, (1993) with some modifications. Cells were lysed with a high salt buffer (0.5 M NaCl, 20 mM Tris/HCl, 10 mM EDTA and 2% SDS) and 100 µL acid washed beads. The cells suspension was vortexed for three min and then centrifuged for five min at 20000 x *g*. The pellet was resuspended in the high salt buffer solution and 200 µL of acid phenol and chloroform was added. Cell suspensions were vortexed for one min and centrifuged for ten min at 15000 x *g*.

The aqueous layer was transferred to a new 1.5 mL reaction tube and 400 µL of chloroform was added. After shaking for a few seconds, the reaction tubes were centrifuged for ten min at 15000 x *g*. Aqueous layer was transferred to a new reaction tube and one mL 100% ice cold isopropanol was added. RNA solution was stored at -20°C overnight to allow for precipitation. The reaction tubes were centrifuged at maximum speed for 10 min at 4°C and the supernatant was discarded. Pellet was dried in a laminar flow cabinet and then resuspended in 100 µL diethyl pyrocarbonate treated dH_2O. Samples were DNAse1 treated to eliminate contamination with genomic DNA. The concentration and purity of the extracted RNA samples were established using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and the integrity of the samples were confirmed through analysis of a Bioanalyzer Chip RNA 7500 series II (Agilent, Santa Clara, CA, USA) according to the manufacturer's instructions. The RNA samples with RNA integrity number (RIN) more than 8, and 280:260 ratios more than 2 were further used for the RNA-sequencing purpose. Library preparation and sequencing was performed by VIB Nucleomics core (Katholieke Universiteit, Leuven, Belgium). Complementary DNA (cDNA) library was generated using TruSeq® Library Prep Kit v2. Paired-end reads were sequenced on the Illumina NextSeq platform.

4.3.7 RNAseq data processing

Low quality reads (< Q20), polyA-reads as well as ambiguous reads (containing N) were removed using FastX 0.0.13 [HannonLab. Fastx-toolkit]. Furthermore, reads shorter than 35 bp were removed with ShortRead 1.16.3 (Morgan et al., 2009) and adapters on the remaining reads were trimmed with cutadapt 1.7.1 (Martin, 2011)

4.3.8 RNAseq data analysis

Annotation of genomic features was performed using the reference genomes of *S. cerevisiae* S288C. Reads from *S. cerevisiae* IWBT Y805 were aligned to the reference genome with TopHat v2.0.13 (Trapnell et al., 2009) and reads that were non-primary mapping or had a mapping quality ≤ 20, were removed. The number of reads in the alignments that overlap with gene features were counted using feature counts 1.4.6. Genes for which all samples had less than one count-per-million were removed. Full quantile normalization using the *EDASeq* package from Bioconductor was applied to correct for sample-specific variation typically introduced by differences in library size and RNA composition. Transcript abundance was measured in Fragments Per Kilobase of exon per Million mapped reads.

4.3.9 Identification and statistical analysis of differentially expressed genes

For the selection of differentially expressed genes statistical modelling was used to design the following experiments:

> Log (Count) = Intercept × β1 + Treatment melatonin only × β2 + Treatment H_2O_2 15 min post stress × β3 +Treatment melatonin and H_2O_2 15 min post stress × β4 + Treatment H_2O_2 30 min post stress × β5 + Treatment melatonin and H_2O_2 30 min post stress × β6 + Treatment H_2O_2 60 min post stress × β7 + Treatment melatonin and H_2O_2 60 min post stress × β8 + Treatment H_2O_2 120 min post stress × β9 + Treatment melatonin and H_2O_2 120 min post stress × β10

For each gene the coefficients β were estimated with the edgeR 3.8.6 package of Bioconductor by fitting a negative binomial generalized linear model (Robinson and Smyth, 2007). Offsets were used to estimate the models. Subsequently, the model estimates were used to compute contrasts of primary interest which were (1) response with melatonin at time t 0 (mel.effect.baseline.t0), (2) response with melatonin at time 15 min (mel.effect.t15minvs0), (3) response with melatonin at time 30 min (mel.effect.t30minvs0), (4) response with melatonin at time 60 min (mel.effect.t60 minvs0), (5) response with melatonin at time 120 min (mel.effect.t120minvs0), (6) response to H_2O_2 stress at 15 min (H_2O_2.15minvsT0), (7) response to H_2O_2 stress at 30 min (H_2O_2.30minvsT0), (8) response to H_2O_2 stress at 60 min (H_2O_2.60minvsT0), (9) response to H_2O_2 stress at 120 min (H_2O_2.120minvsT0), (10) Melatonin effect at time 15 min (melvsH_2O_2.effect.t15min) to find genes that have responded differently to the melatonin and the H_2O_2 at 15 min, (11) Melatonin effect at time 30 min (melvsH_2O_2.effect.t30min) to find genes that have responded differently to the melatonin and the H_2O_2 at 30 min, (12) Melatonin effect at time 60 min (melvsH_2O_2.effect.t60min) to find genes that have responded differently to the melatonin and the H_2O_2 at 60 min, (13) Melatonin effect at time 120 min (melvsH_2O_2.effect.t120min) to find genes that have responded differently to the melatonin and the H_2O_2 at 120 min, (14) Melatonin effect at all time points (treatment.effect.all) to find genes that have responded differently to the melatonin and the H_2O_2 at all time points after stress induction. The differential expression was tested with a generalized linear model likelihood ratio test, also implemented in the edgeR 3.8.6 package. The resulting *p*-values were corrected for multiple testing to control the false discovery rate (FDR) (Benjamini and Hochberg, 1995). Genes with an absolute $\log_2$-ratio larger than one and an adjusted *p*-value < 0.05 (1.1E-03) were considered differentially expressed.

4.4 Results

4.4.1 Determination of nitrogen limiting concentration

Nitrogen was selected as the limiting nutrient in the continuous culture experiments as melatonin synthesis is linked to amino acid and nitrogen metabolism in various organisms (Reiter, 1991; Sprenger et al., 1999; Murch et al., 2000; Iriti et al., 2006). These culture conditions were intended to investigate the impact of nitrogen sources on melatonin production by *S. cerevisiae* IWBT Y805. Several concentrations of $(NH_4)_2SO_4$ were tested to determine the limiting concentration in continuous culture. Experimental conditions were established by feeding the bioreactor with minimal medium (80 g/L glucose) after approximately 30 h of batch cultivation (late exponential phase) at a dilution rate 0.264/h to prevent washout of cultures. Samples were taken once cultures reached steady state and ammonia analysis performed to determine nitrogen levels in the medium.

Table 4.1. Residual ammonia concentrations (mean ± range of duplicate measurements) under steady conditions in the bioreactor at 0.264/h dilution rate fed with glucose (80 g/L) minimal medium containing various $(NH_4)_2SO_4$ concentrations

	$(NH_4)_2SO_4$ (g/L) added to bioreactor		
	0.3	0.6	5
Concentrations of ammonia (mg/L) in steady state cultures	0.9 ± 0.03	59.5 ± 1.6	70 ± 2.1
Maximum specific growth rate (/h) [a]	0.32	0.336	0.335

[a] Maximum specific growth rate determined in batch mode before start of medium pump at a dilution rate of 0.264/h

The data in this study showed that at 0.3 g/L $(NH_4)_2SO_4$ the ammonia concentrations at steady state were close to zero (0.9 mg/L). When the concentration of $(NH_4)_2SO_4$ in feed medium was increased to 0.6 and 5 g/L, the residual ammonia levels at steady state were 59.5 and 70 mg/L respectively (Table 4.1), suggesting that other factors had become limiting. Consequently, 0.3 g/L was selected as the limiting concentration of $(NH_4)_2SO_4$. The maximum growth rate was of cultures at this concentration of $(NH_4)_2SO_4$ was 0.32/h (Fig 4.1). Similar conditions were found by Kenyon et al. (1986).

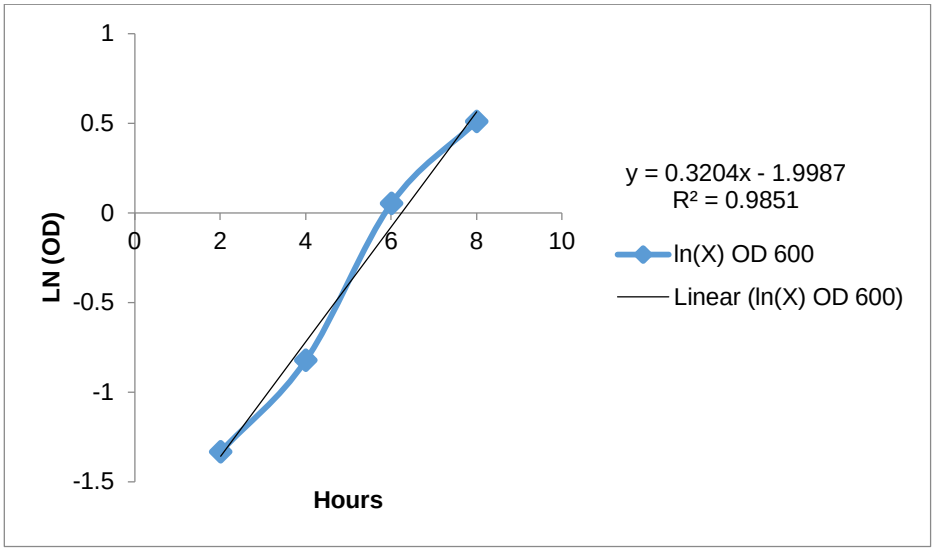

Figure 4.8: Maximum growth rate of *S. cerevisiae* cultured in minimal medium containing 0.3 g/L $(NH_4)_2SO_4$.

4.4.2 Response of *S. cerevisiae* to oxidative stress in continuous culture

Hydrogen peroxide was used to induce oxidative stress in the experiments and a sublethal concentration of the stressor was determined in steady state cultures. A concentration of 1 mM H_2O_2 was lethal to the cells, and the cultures did not recover from the stress (Fig 4.2A), while a concentration of 0.6 mM H_2O_2 led to a short growth arrest followed by recovery to near pre-stress growth rates within 120 min (Fig 4.2B). Thus, this concentration was used in subsequent experiments.

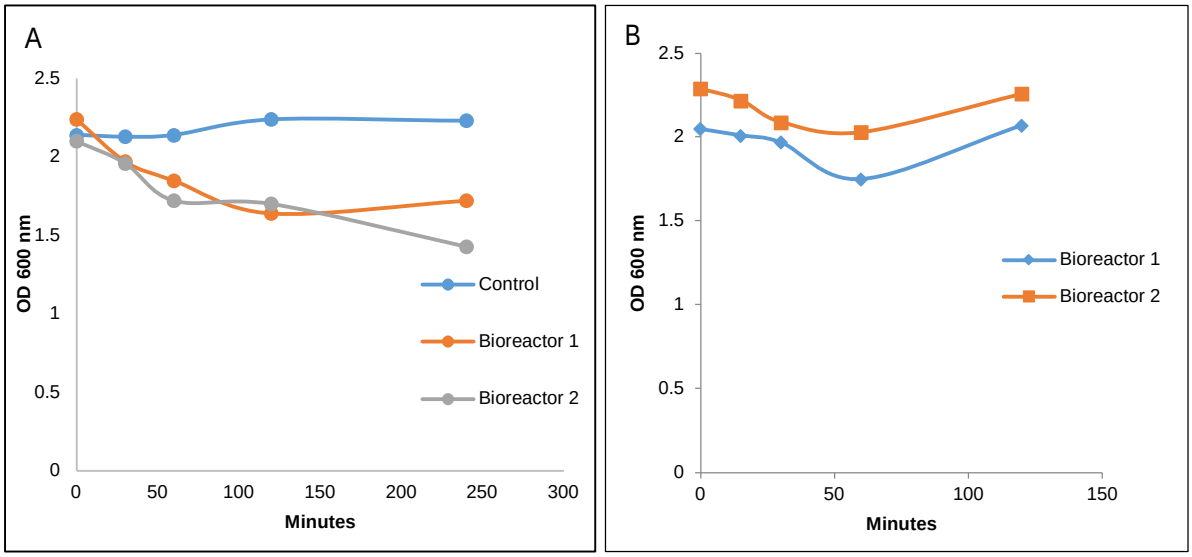

Figure 4.2: Response of *S. cerevisiae* (IWBT Y805) to (A) 1 mM and (B) 0.6 mM H_2O_2 stress in nitrogen limited continuous culture at a 0.264/h dilution rate. Stress was applied once the cultures were in a steady state, which was determined by stable OD_{600} readings over three residence times.

4.4.3 Response of *S. cerevisiae* to oxidative stress in the presence of melatonin in continuous culture

Oxidative stress was induced in melatonin treated and untreated cultures and the impact of melatonin on yeast viability post exposure to H_2O_2 stress monitored over a two-hour period. Melatonin treated cultures recovered faster from the stress when compared to the untreated cells (Fig 4.3).

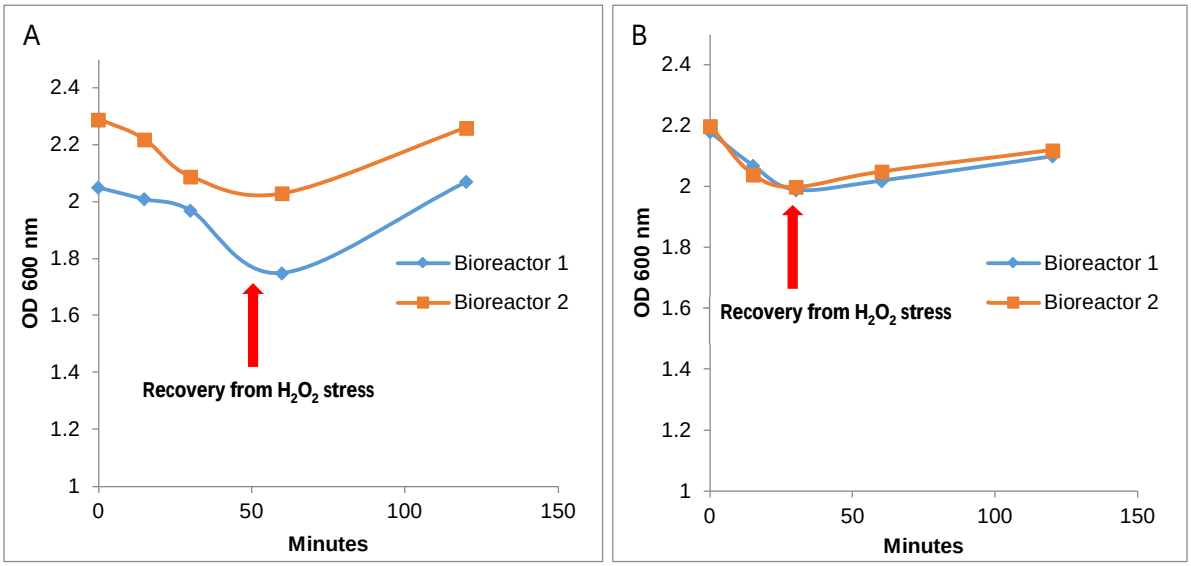

Figure 4.3: Impact of 0.6 mM H_2O_2 induced stress (added at time 0) on growth of *S. cerevisiae* IWBT Y805 in (A) absence of and (B) presence of 300 µM melatonin. Experiments were conducted in continuous culture at a dilution rate of 0.264/h. Stress was applied once cultures were in a steady state which was determined by stable OD_{600} readings over three residence times.

While the biomass (as measured by OD_{600}) was slightly different between the two bioreactors (2.05 vs 2.3) when melatonin was absent in continuous culture, the recovery response to H_2O_2 was identical in both cases. The levels of glucose and ammonium were monitored throughout the experiment. Prior to addition of H_2O_2, ammonium concentrations were below detection limits in the continuous culture (Table 4.2) confirming that the cultures were indeed under nitrogen limitation. Ammonium concentrations increased after addition of stressor as expected due to reduction in cell absorbance. The initial glucose levels were approximately 65 g/L in the continuous culture and decreased slightly immediately after the addition of the H_2O_2, suggesting a rapid metabolic response to the stress. Thereafter, glucose levels increased slightly to above 70 g/L, reflecting the initial decline in cell absorbance during the recovery phase. At 120 min, glucose levels decreased to near pre-stress levels as cell absorbance readings increased to pre-stress levels.

Table 4.2: Impact of H_2O_2 induced oxidative stress on glucose and ammonium concentrations (mean ± range of duplicate measurements) in glucose (80 g/L) $(NH_4)_2SO_4$ (0.3 g/L) minimal medium under the continuous culture at 0.264/h dilution rate

	0 min	15 min	30 min	60 min	120 min
Glucose (g/L)	65.9±1.1	61.7±7.4	71.4±1.3	71.3±0.2	67.6±3.6
Ammonium (mg/L)	ND [a]	0.05	2.1±0.4	5.9±0.4	7.8±0.2

[a] Below detection limit of assay for accurate quantification

The supernatants from the two treatment groups were analysed by LCMS/MS for the detection and quantification of metabolites in the melatonin biosynthetic pathway i.e. tryptophan, melatonin, 5-hydroxytryptophan, serotonin, 5-methoxytryptamine, 3-indole acetic acid, tryptophol and 6-hydroxymelatonin. Low endogenous melatonin concentrations were found in the supernatant of all the samples without melatonin supplementation and this was the only metabolite that was quantifiable in these samples. Apart from melatonin, the only metabolite quantifiable in the melatonin supplemented samples was 6-hydroxymelatonin. Of the initial 300 µM melatonin concentration added to the bioreactor, approximately 1.7 µM remained in the medium at time 0 pointing to rapid melatonin uptake. Upon H_2O_2 addition, melatonin concentration increased pointing to possible reduction in cell uptake or a release from previously accumulated melatonin. The break-down product of melatonin, 6-hydroxymelatonin, only quantifiable in the samples supplemented with melatonin, was found to be at approximately half of the levels of melatonin (Table 4.3).

Table 4.3: Impact of 300 µM melatonin supplementation during H_2O_2 induced oxidative stress on residual melatonin and 6-hydroxymelatonin concentrations (mean ± range of duplicate measurements) in continuous culture (0.264/h dilution rate)

	Melatonin (µg/L)		6-hydroxymelatonin (µg/L)	
Time (min)	Melatonin treated	Untreated	Melatonin treated	Untreated
0	397±47	0.24±0.08	144±60	ND[a]
15	397±11	0.13±0.03	255±17	ND
30	470±21	0.09±0.03	196±20	ND
60	439±54	0.08±0.02	173±50	ND
120	452±32	0.07±0.002	219±21	ND

[a] Below detection limit of assay for accurate quantification

4.4.4 Overview of transcriptional data

RNA extractions were performed on two biological repeats of melatonin treated and untreated samples before (0 min) and (15, 30, 60 and 120 min) after the induction of H_2O_2 stress. After removing the low-quality reads, the remaining data was aligned to the *S. cerevisiae* S288C reference genome. Over 90% of the total reads mapped to the S288C genome (Table S4.1). Principal Component Analysis (PCA) (Fig S4.1) performed on normalized transcriptome data showed that the samples clustered together firstly by time point, then per treatment. Control samples of melatonin at 60 min however did not cluster together and were not included in further comparisons. The same observation applied to H_2O_2 stress-induced samples of melatonin at 60 min (Mel 60 min), however these samples were included in some further analysis since they were useful for hypothesis development. The interaction effect between the stress induction effect and the melatonin treatment effect was performed by using a Nested interaction model (ANOVA like approach). This analysis captured significantly differentially expressed genes (DEG) between any of the groups (i.e. genes that respond to the treatment at any time: 15 min, 30 min, 60 min and 120 min with and without melatonin). Genes with an absolute $\log_2$-ratio larger than one and an adjusted *p*-value < 1.1E-03 were considered differentially expressed and genes showing significant interactions (FDR-value less than 0.05) were filtered out. The data will be submitted to the data depository (http://www.ncbi.nlm.nih.gov).

4.4.5 Transcriptional response to melatonin in the absence of H_2O_2 induced stress

Melatonin supplementation to steady state cultures of *S. cerevisiae* differentially expressed 579 genes (347 up-regulated and 232 down-regulated) (absolute $\log_2$-ratio > or < 1) in the absence of oxidative stress (Table S4.2). Some of the highly over-expressed genes were involved in biotin biosynthesis (*BIO5*), vitamin B6 biosynthesis (*SNO1*), uptake of some amino acids (*BAP3*) and RNA processing (*RRN11, UTP13, UTP9, BFR2* and *NRP1*) (Fig S4.2 A). Melatonin caused reduced expression of some genes encoding amino acid transporters (*DIP5*), and enzymes involved in arginine metabolism (*CAR1*) and proline utilization (*PUT1*) (Fig S4.2 B).

Gene Ontology (GO) analysis was performed on the differentially expressed genes using the Gene Ontology enRIchment anaLysis and visualization (GOrilla) tool (Eden et al., 2007; Eden et al., 2009). This platform separated GO terms into three categories (biological process, molecular function and cellular component) (Fig 4.4). The biological processes

enriched with up-regulated genes were metabolic processes and the genes highlighted include *MET28* (Log FC 1.1), a transcription factor which regulates sulphur metabolism, as well as its targets *MET1, MET2, MET14, MET16* and *MET17* (Log FC 1.7, 2, 1.1, 1 and 1.1, respectively). Molecular functions enriched by up-regulated genes are associated with binding of compounds whereas cellular components were associated with cell structures. Analysis of down-regulated genes revealed that catabolic process and amino acid transmembrane transport were the enriched biological processes. Furthermore, molecular functions enriched involve ubiquitin-ubiquitin ligase activity and transporter activities, while the cellular components enriched were associated with cell membrane.

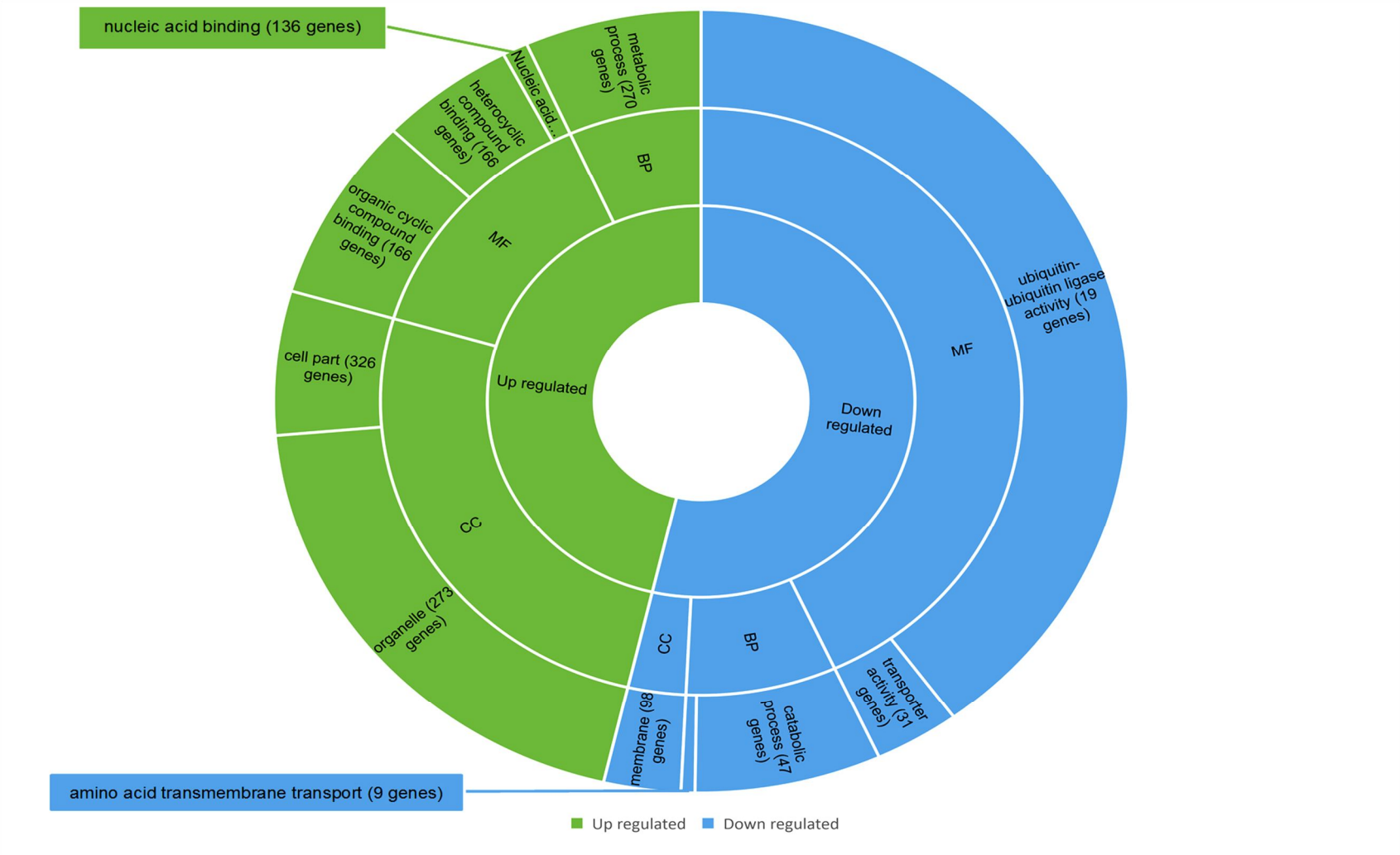

Figure 4.4: Sunburst chart of enriched GO terms (Biological Processes (BP), Molecular Function (MF) and Cellular Component (CC)) in melatonin (300 µM) treated *S. cerevisiae* cultured in nitrogen-limited chemostat. The size of the outer section panels of the figure is proportional to the significance degree (p-value) of the function.

4.4.6 Transcriptional response to H_2O_2 induced stress in the presence and absence of melatonin

H_2O_2 stress responses were monitored after addition of the stressor. A large number of genes responded in all cases, with the H_2O_2 only treatment showing a higher number of differentially expressed genes in comparison to H_2O_2 treatment in the presence of melatonin (Table 4.4). Over time, the number of differentially expressed genes decreased in line with yeast recovery from the stress. Furthermore, the presence of melatonin alleviated the number of genes responding to H_2O_2 stress compared to melatonin untreated cultures

Table 4.4: Distribution of differentially expressed genes in the melatonin + H_2O_2 treatment and H_2O_2 only treatment across the experimental time course

	15 min		30 min		60 min		120 min	
	Melatonin + H_2O_2	H_2O_2 only	Melatonin + H_2O_2	H_2O_2 only	Melatonin + H_2O_2	H_2O_2 only	Melatonin + H_2O_2	H_2O_2 only
Up-regulated	591	748	721	743	38	511	58	531
Down-regulated	602	752	748	769	6	485	47	459
Total regulated	1193	1500	1469	1512	44	996	105	990

Venn diagram analysis was performed on the up- and down-regulated genes across the timepoints to identify common genes regulated by melatonin and H_2O_2 (Figs. 4.5 and 4.6). In the comparison of the genes differentially expressed at 15 and 30 min H_2O_2 treatment (with and without melatonin) with melatonin baseline, the largest number of overlapping genes (265 genes) was in response to the H_2O_2 stress irrespective of melatonin treatment. The second and third largest gene overlaps were between the 15 and 30 min timepoints in the H_2O_2 only treatment and the melatonin with H_2O_2 (M+H) treatment with 112 and 102 genes, respectively. These genes therefore appear to respond in a melatonin dependent manner to the stress. At the 15 min timepoint 30 genes overlapped in the melatonin treated and untreated samples whereas 43 genes overlapped at the 30 min timepoint. Comparison of the genes differentially expressed at 15 and 30 min H_2O_2 treatment (with and without melatonin) with melatonin baseline found 23 commonly up-regulated genes (Fig 4.5A). There was no overlap of genes in the comparison of the 60 min, 120 min and melatonin baseline treatments (Fig 4.5B). The largest gene overlap (213 genes) was between melatonin baseline and the 60 min and 120 min H_2O_2 treatment and 93 genes overlapped between the H_2O_2 120 min timepoint and melatonin baseline suggesting that cells recovered from stress at the timepoint. Only 23 genes and 14 genes overlap at the 60 min and 120 min timepoints respectively.

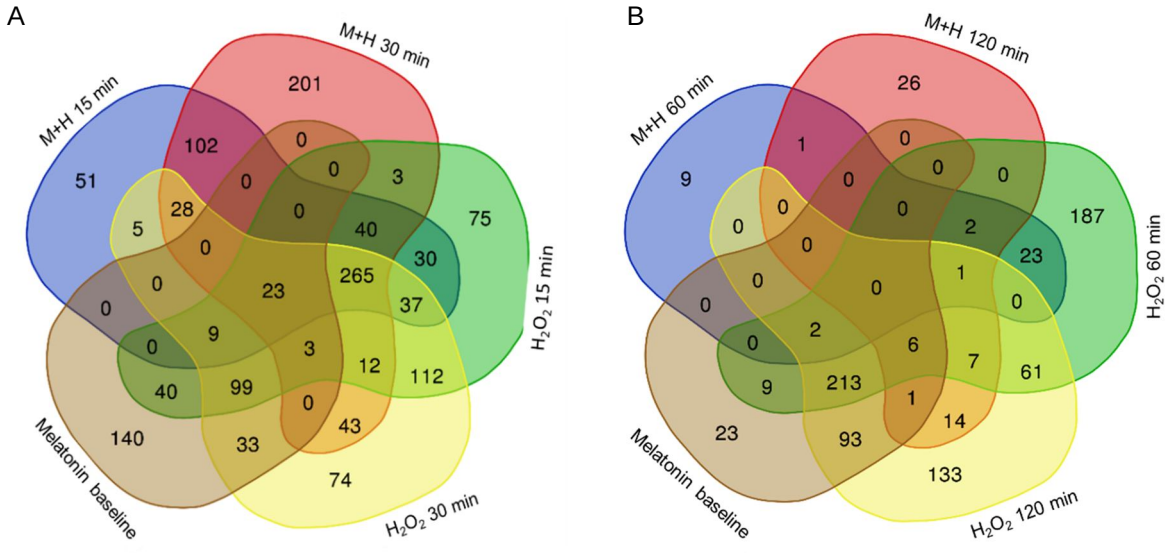

Figure 4.5: Venn diagrams summarising the distribution and overlaps of differentially expressed genes up-regulated in the melatonin only treatment, melatonin + H_2O_2 treatment (M+H) and H_2O_2 only treatment across the experimental time course. (A) Comparison of the 15, 30 min H_2O_2 treatments and melatonin baseline. (B) Comparison of the 60, 120 min H_2O_2 treatments and melatonin baseline.

Gene overlaps of the down-regulated genes mirrored the pattern observed with the up-regulated genes. The comparison of the 15 and 30 min H_2O_2 treatment (with and without melatonin) with melatonin baseline revealed an overlap of 22 genes whereas a comparison of just the 15 min and 30 min timepoints found an overlap of 174 genes. In the M+H treatment 206 genes overlapped between the 15 and 30 min timepoint and 220 genes overlapped in the H_2O_2 treatment at the same timepoints. At the 15 min timepoint 26 genes overlapped in the melatonin treated and untreated samples and 31 genes overlapped at the 30 min timepoint (Fig 4.6A). At the later timepoints (60 and 120 min) there were no genes that overlapped in the H_2O_2 treated samples. The largest overlap was between the 60 min and 120 min H_2O_2 samples and melatonin baseline (106 genes) and 72 genes overlapped between the H_2O_2 2 h timepoint and melatonin baseline. There were only two genes that overlapped at 60 min timepoint and 16 genes overlapped at the 120 min timepoint (Fig 4.6B).

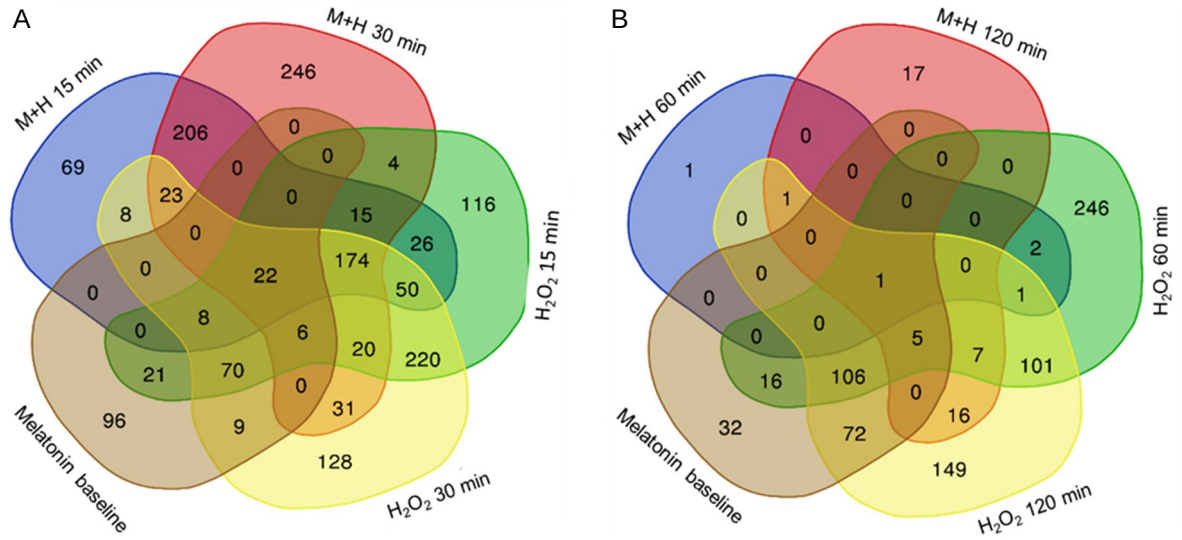

Figure 4.6: Venn diagrams summarising the distribution and overlaps of differentially expressed genes down-regulated in the melatonin only treatment, melatonin + H_2O_2 treatment (M+H) and H_2O_2 only treatment across the experimental time course. (A) Comparison of the 15, 30 min H_2O_2 treatments and melatonin baseline. (B) Comparison of the 60, 120 min H_2O_2 treatments and melatonin baseline.

GO enrichment analysis was performed on overlapping differentially expressed genes. The 23 genes commonly up-regulated in the comparison of the 15 and 30 min H_2O_2 treatment (with and without melatonin) with melatonin baseline are involved in organonitrogen compound biosynthetic process (14%) and small molecule metabolic process (15%). The 265 genes that overlap at the 15 and 30 min timepoints were mainly involved in stress response (Fig 4.5A) (Table S4.3). The 213 commonly up-regulated genes in the H_2O_2 only (60 and 120 min) and melatonin baseline treatments were mainly involved in metabolic processes (Fig 4.5B) (Table S4.3). The 22 down-regulated genes overlapping between the 15 and 30 min H_2O_2 treatment (with and without melatonin) with melatonin baseline were mainly involved biosynthetic process (Fig 4.6A) (Table S4.4). The largest overlap was between the 60 min and 120 min H_2O_2 samples and melatonin baseline (106 genes). These genes were involved with small molecule metabolic processes and transmembrane transport (Table S4.4).

4.4.7 Impact of melatonin on transcriptional response of *S. cerevisiae* to oxidative stress

GO enrichment analysis of the DEG's in the presence and absence of melatonin showed that the transcriptional response of the cultures to the oxidative stress changed during the stress period (Jamieson, 1998). The results are summarized in Fig 4.7. Processes that were enriched by up-regulated DEG's in the first 30 min were related to cellular response to oxidative stress (Tables S4.5). At the 60 min time point processes related to the uptake of glucose, amino acid biosynthesis and cellular component biogenesis were enriched (Table S4.6). Processes enriched at the 120 min time point were associated with nitrogen compound metabolic process, organic substance metabolic process, cellular respiration and RNA metabolic process (Table S4.6). The enrichments at the last time point are similar to biological processes enriched at melatonin baseline. Down-regulated genes enriched biological processes associated with transmembrane transport and RNA processing (Table S4.7). In the first 30 min of stress, nucleotide translation and modification processes were down-regulated to prevent protein synthesis in this potentially error prone condition. At the 60 min and 120 min timepoints down-regulated processes resemble melatonin baseline enriched processes.

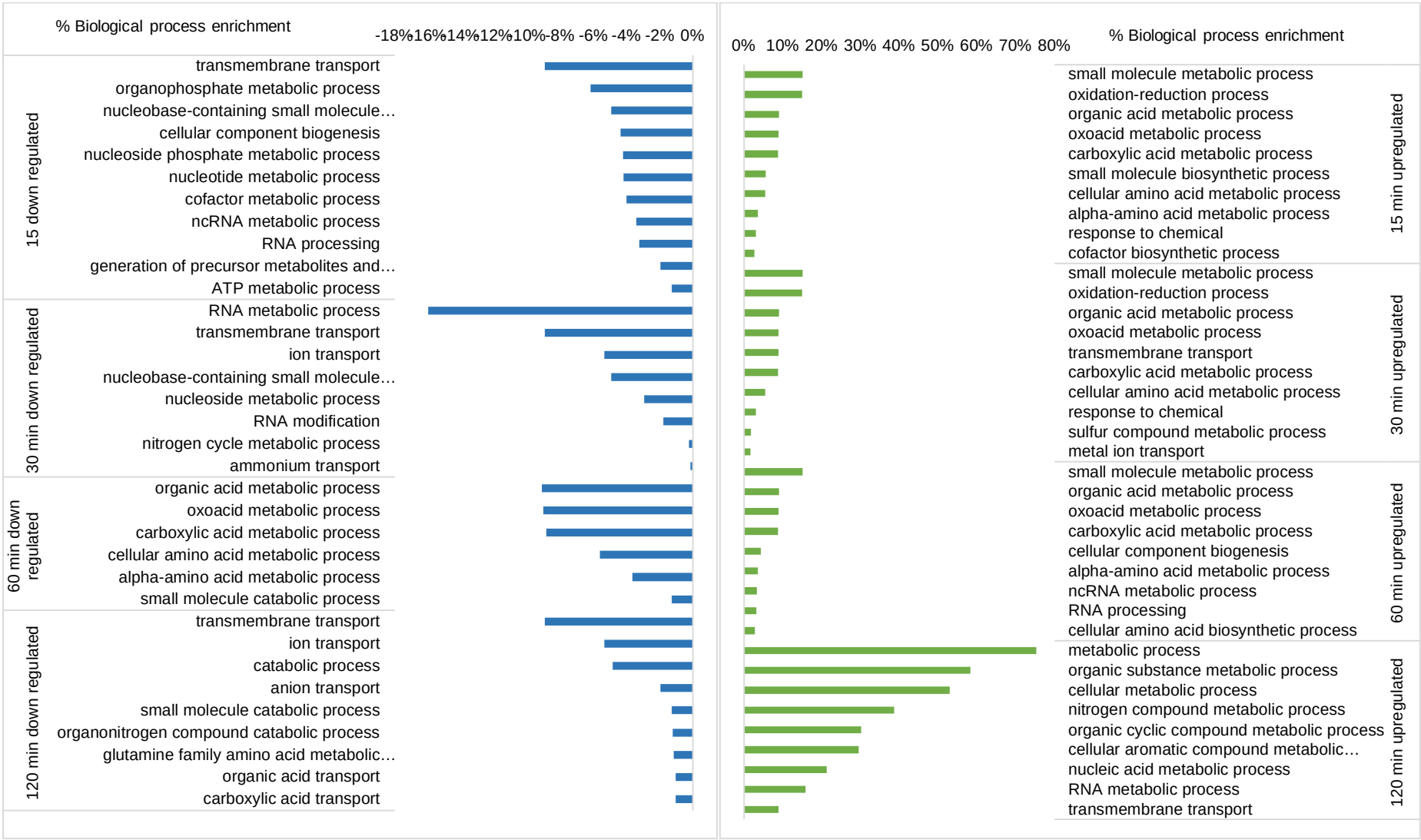

Figure 4.7: Biological process enrichment from Gene ontology (GO) analysis of differentially expressed genes up- (■) and down- (■) regulated (fold change ≥1; p-value < 0.05) in H_2O_2 (0.6 mM) stressed *S. cerevisiae* cells in presence or absence of melatonin (300 µM). Percentages are calculated in relation to the total genes involved in each biological process. Positive values indicate up-regulation and negative values indicate down-regulation.

The transcriptome of the melatonin treated cultures during oxidative stress at each timepoint was compared to the transcriptome of the untreated cultures at the corresponding timepoint to assess the impact of melatonin on the transcriptional response of *S. cerevisiae* to oxidative stress. This filtering focused our analysis on the DEGs in Fig 4.8 and these DEGs were analysed for enriched GO terms at the four sampling time points.

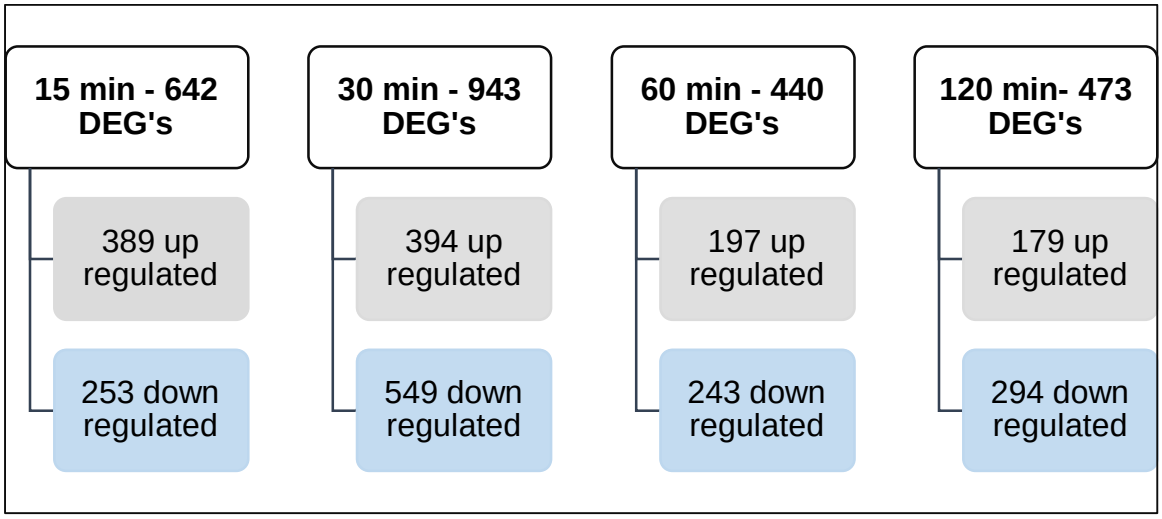

Figure 4.8: Number of differentially expressed genes (DEG's) in *S. cerevisiae* in response to melatonin during H_2O_2 induced oxidative stress in continuous culture. Time points correspond to sample timepoints.

GO enrichment analysis of genes up-regulated at 15 min showed that transmembrane transport, glutamine family amino acid metabolic processes, steroid biosynthetic processes and catabolic process were enriched. The genes up-regulated in the steroid biosynthetic process were *ERG4, ERG9, ERG3, ERG26* and *ERG28* (Log_2 fold change 1.1, 1.03, 1.2, 1.2, and 1.9 respectively). At the 30-min time point the enriched biological processes were glutamine family amino acid metabolic processes, ion transport, cellular and organic nitrogen compound catabolic processes, while after 60 min it was mainly processes associated with cell cycle, drug metabolism, glucose metabolism, ADP metabolism and alpha amino acid metabolism. At the 120 min time point up-regulated DEG's enriched processes associated with glutamine family amino acid metabolism, nitrogen cycle metabolism and small molecule metabolism. Down-regulated DEG's across all time points were associated with metabolic processes, RNA metabolic process, cellular component biogenesis and vitamin metabolism (Fig 4.9).

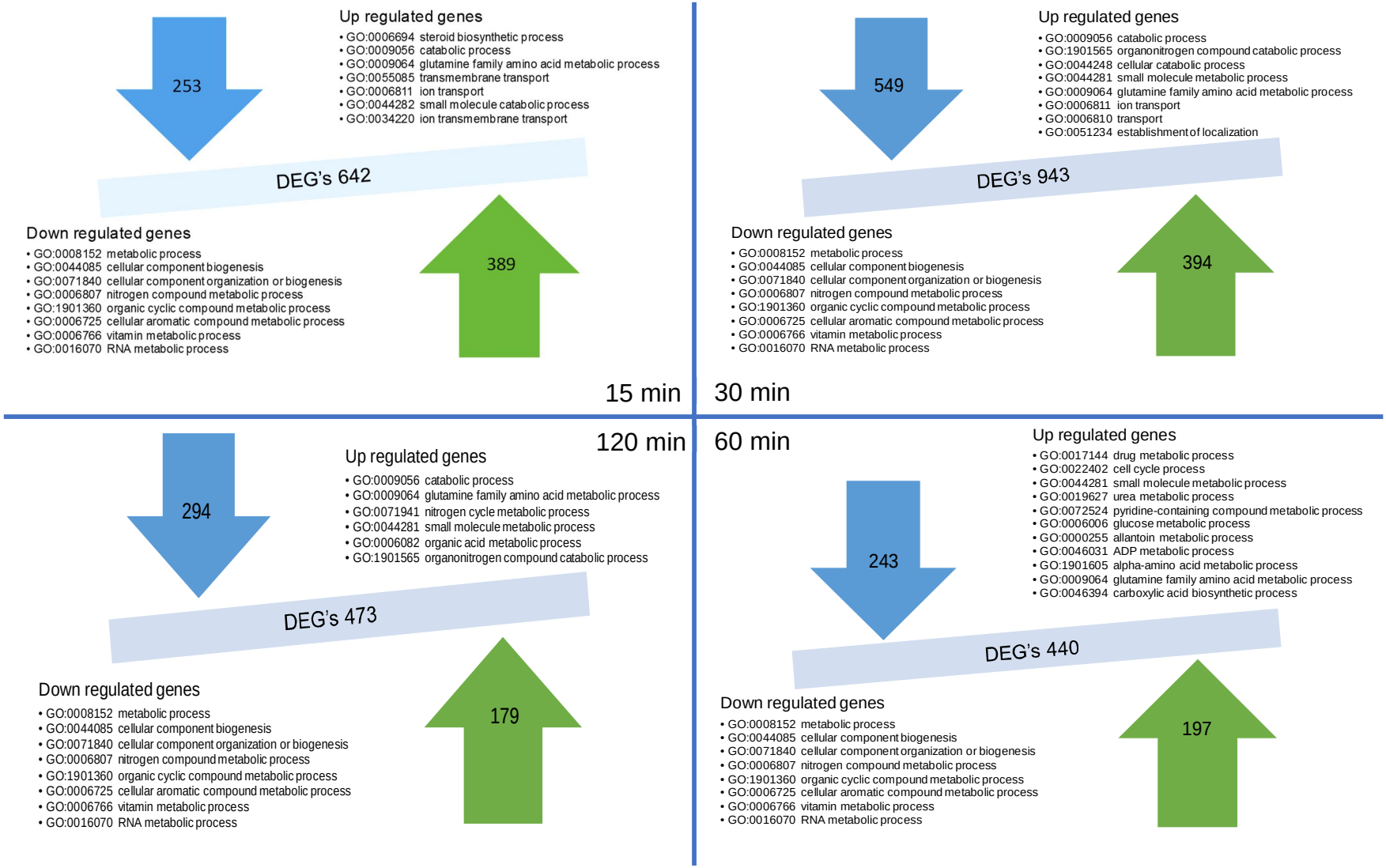

Figure 4.9: Biological process enrichment from Gene ontology (GO) analysis of differentially expressed genes up- (■) and down- (■) regulated (fold change ≥1; p-value < 0.05) in H_2O_2 (0.6 mM) in response to 300 µM melatonin treatment during oxidative stress.

4.4.8 Transcriptional response to oxidative stress

Transcriptional factors *YAP1, SKN7* and *MSN2/4* regulate the expression of genes involved in redox homeostasis. In this study the transcription factors were up-regulated during the oxidative stress (Fig 4.10) and the presence of melatonin did not have an impact on the differential expression of *YAP1. SKN7* was up-regulated in the H_2O_2 only treatment. The expression of *MSN2* and *MSN4* transcription factors was higher in the presence of melatonin. *MSN2* was up-regulated in both treatments with the first 30 min but the expression levels were higher in the melatonin treatment. At the 60- and 120-min time points, this transcription factor was down-regulated but to a lesser extent in the melatonin treated samples. *MSN4* transcription factor was down-regulated in both treatments at 15 min but once again to a lesser extent in melatonin treated samples. At the 30 min timepoint it was up-regulated only in the melatonin treatment. This transcription factor was up-regulated in both treatments at 60-min but the expression levels were higher in the melatonin treatment. At the last sampling point *MSN4* was up-regulated in melatonin treatment and down-regulated in the H_2O_2 only treatment.

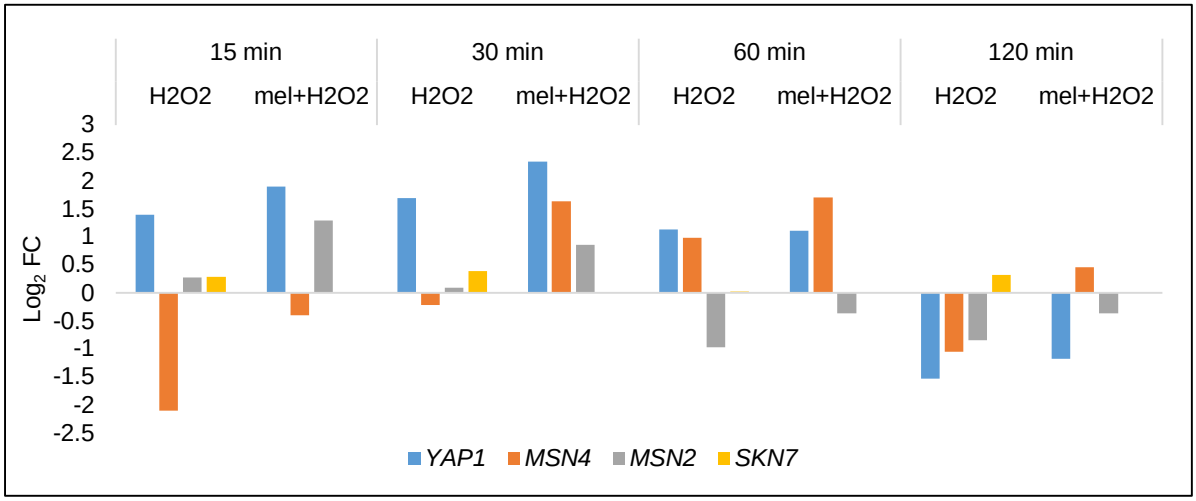

Figure 4.10: Expression of transcription factors in the presence and absence of melatonin during H_2O_2 induced oxidative stress. H_2O_2 fold change relative to expression in the absence of H_2O_2. Melatonin + H_2O_2 fold change relative to expression in the presence of melatonin and absence of H_2O_2.

The expression profile of oxidative stress response (OSR) genes over time in the presence and absence of melatonin was analysed. This dataset was compared with literature to identify OSR genes that were differentially expressed in this experiment. Genes were filtered based only on the p-value (< 0.05). We identified twenty-three genes that were differentially expressed in response to H_2O_2 in the absence and presence of melatonin (Fig 4.11) (Table S4.8). These genes included superoxide dismutases, catalases, thioredoxins,

glutathione, glutaredoxin family. The highest expression of these genes occurred in the first 30 min after addition of stress and decrease thereafter.

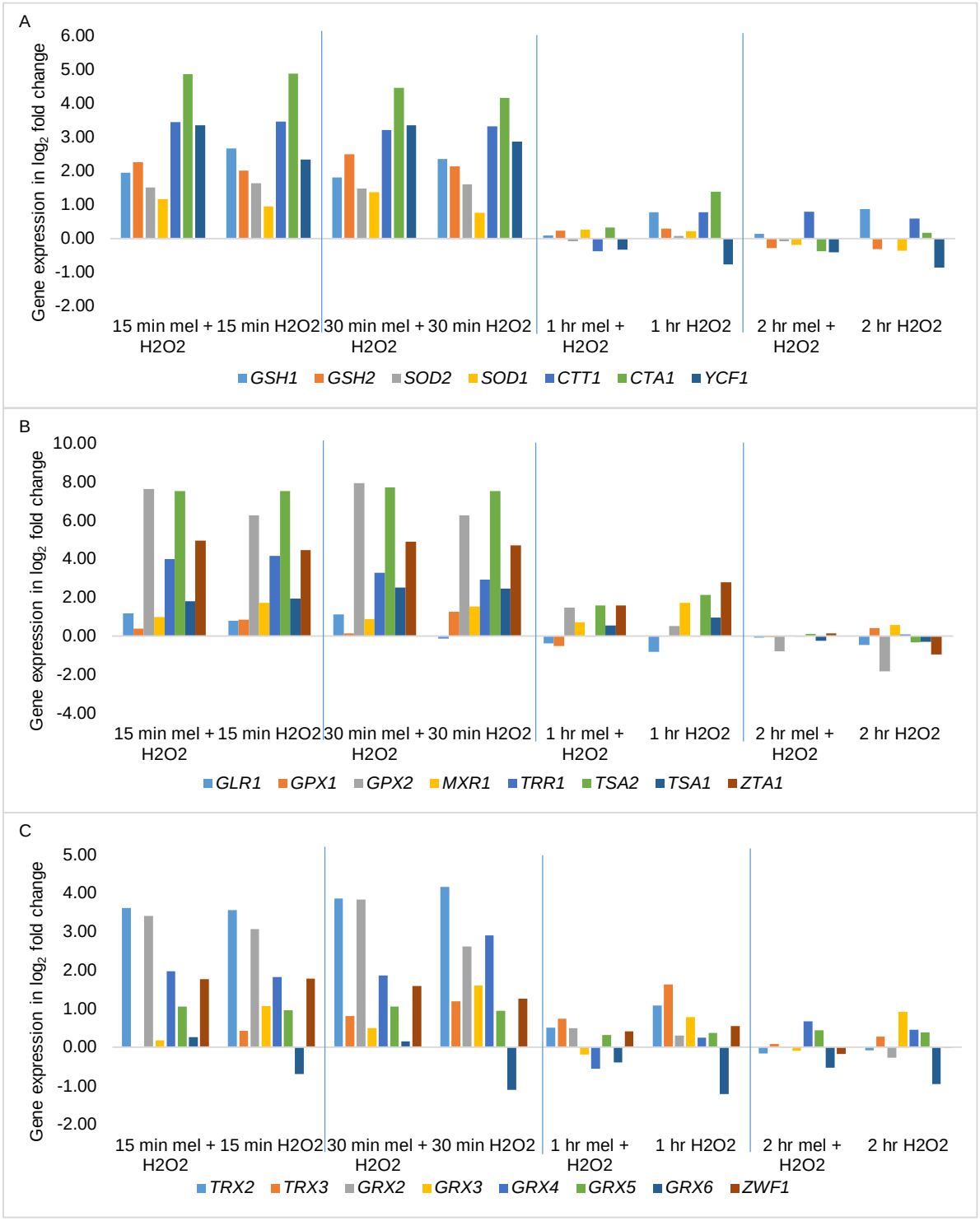

Figure 4.11: Differential expression of genes involved in oxidative stress relative to unstressed *S. cerevisiae* cells in the presence and absence of melatonin (Log_2 fold change).

The OSR genes reported in this study were compared to other studies investigating the transcriptional response of *S. cerevisiae* to oxidative stress. Of the 23 genes listed in Table 4.5, 16 genes were reported in other studies (Gasch *et al.*, 2000; Koerkamp *et al.*, 2002; Shenton *et al.*, 2006; Vazquez et al., 2018). Application of oxidative stress in this study also induced the expression of two glutathione reductase encoding genes (*GRX5, GRX6*), a methionine-S-sulfoxide reductase (*MXR1*), cytoplasmic thioredoxin peroxidase (*TSA2*), NADPH-dependent quinone reductase (*ZTA1*) and glucose-6-phosphate dehydrogenase (*ZWF1*).

Of these 23 genes, six were differentially expressed in response to melatonin when compared to gene expression in the absence of melatonin (Fig 4.11). The expression of *GRX3* (glutathione oxireductase; at 15 and 30 min) (Fig 4.11C) and *GPX1* (glutathione peroxidase; at 30 min) (Fig 4.11B) was decreased by melatonin. *GRX6* (monothiol glutaredoxin; at 15 and 30min) (Fig 4.11C), *GPX2* (glutathione peroxidase), *GLR1* (glutathione oxireductase; at 30min) (Fig 4.11B) and *YCF1* (vacuolar glutathione S-conjugate transporter; at 15 and 30 min) (Fig 4.11A) were up-regulated in the presence of melatonin.

Table 4.5: List of genes up- and down-regulated by oxidative stress at least one-fold, their functional description and comparison with previous reports

Gene	Fold change	Description	Overlapping with			
			(Shenton et al., 2006)	Vazquez et al 2018	(Gasch et al., 2000)	(Koerkamp et al., 2002)
CTA1	4.9	Catalase A - breaks down hydrogen peroxide in the peroxisomal matrix formed by acyl-CoA oxidase (Pox1p) during fatty acid beta-oxidation			☑	
CTT1	3.5	Cytosolic catalase T which has a role in protection from oxidative damage by hydrogen peroxide.	☑	☑	☑	
GLR1	1.2	Cytosolic and mitochondrial glutathione oxidoreductase - converts oxidized glutathione to reduced glutathione; cytosolic Glr1p is the main determinant of the glutathione redox state of the mitochondrial intermembrane space.				☑
GPX1	1.3	Phospholipid hydroperoxide glutathione peroxidase - induced by glucose starvation that protects cells from phospholipid hydroperoxides and nonphospholipid peroxides during oxidative stress		☑	☑	
GPX2	7.9	Phospholipid hydroperoxide glutathione peroxidase; protects cells from phospholipid hydroperoxides and nonphospholipid peroxides during oxidative stress	☑		☑	☑

Gene	Fold change	Description	Overlapping with			
			(Shenton et al., 2006)	Vazquez et al 2018	(Gasch et al., 2000)	(Koerkamp et al., 2002)
GRX3	1.6	Glutathione-dependent oxidoreductase - hydroperoxide and superoxide-radical responsive; monothiol glutaredoxin subfamily member along with Grx4p and Grx5p; protects cells from oxidative damage.				
GRX4	2.9	Glutathione-dependent oxidoreductase - protects cells from oxidative damage.	☑			
GRX5	1.05	Glutathione-dependent oxidoreductase - hydroperoxide and superoxide-radical responsive.				
GRX6	-1.2	Cis-golgi localized monothiol glutaredoxin, binds Fe-S cluster - involved in the oxidative stress response				
GSH1	2.8	Gamma glutamylcysteine synthetase - catalyzes the first step in glutathione (GSH) biosynthesis; expression induced by oxidants, cadmium, and mercury				☑
GSH2	2.5	Glutathione synthetase - catalyzes the ATP-dependent synthesis of glutathione (GSH) from gamma-glutamylcysteine and glycine; induced by oxidative stress and heat shock				☑
MXR1	1.7	Methionine-S-sulfoxide reductase; involved in the response to oxidative stress; protects iron-sulphur clusters from oxidative inactivation along with MXR2; involved in the regulation of lifespan.		☑		
SOD1	1.4	Cytosolic copper-zinc superoxide dismutase - detoxifies superoxide; phosphorylated by Dun1p and enters the nucleus under oxidative stress to promote transcription of stress response genes;			☑	☑
SOD2	1.6	Mitochondrial manganese superoxide dismutase - protects cells against oxygen toxicity.	☑			
TRR1	4.1	Cytoplasmic thioredoxin reductase. Key regulatory enzyme that determines redox state of thioredoxin system, which acts as a disulphide reductase system and protects cells against both oxidative and reductive stress.	☑			☑
TRX2	4.2	Cytoplasmic thioredoxin isoenzyme. Part of thioredoxin system which protects cells against oxidative and reductive stress.		☑	☑	☑

Gene	Fold change	Description	Overlapping with			
			(Shenton et al., 2006)	Vazquez et al 2018	(Gasch et al., 2000)	(Koerkamp et al., 2002)
TSA1	2.5	Thioredoxin peroxidase - acts as both ribosome-associated and free cytoplasmic antioxidant				☑
TSA2	7.5	"Stress inducible cytoplasmic thioredoxin peroxidase. Cooperates with Tsa1p in the removal of reactive oxygen, nitrogen and sulphur species using thioredoxin as hydrogen donor.				
YCF1	3.34	Vacuolar glutathione S-conjugate transporter. Plays a role in detoxifying metals (Cd, Hg, As) and transports GSSG that is not immediately reduced in cytosol to vacuole	☑			
ZTA1	4.9	NADPH-dependent quinone reductase				
ZWF1	1.8	Glucose-6-phosphate dehydrogenase (G6PD) - catalyzes the first step of the pentose phosphate pathway; involved in adapting to oxidative stress				

4.4.9 Overview of the impact of melatonin in metabolic pathways

Data of the DEG's in response to melatonin at baseline and across treatment timepoints was overlaid on the Biocyc Omics Dashboard to visualise the impact of melatonin on metabolic and signalling pathways. Averages of the Log_2 fold change of genes enriched in each pathway are represented in Fig 4.12 based on data presented in Table S4.9.

In the stimulus response category, the detoxification pathways were enriched during H_2O_2 induced oxidative stress in the presence of melatonin although the fold changes were not exceptionally high. The energy metabolism class comprises glycolysis, tricarboxylic acid (TCA), pentose phosphate pathway (PPP), fermentation, chemoautrophic energy and other metabolic pathways. In the absence of oxidative stress, melatonin supplementation enriched the TCA and PPP. Overall the PPP was down-regulated slightly in the presence of melatonin after induction of H_2O_2 stress but there was up-regulation of transaldolase (*TAL1*), transketolase (*TKL1*), glucose-6-phosphate dehydrogenase (ZWF1) and 6-phosphogluconate dehydrogenase, decarboxylating 1 (*GND1)*. Enrichment of glycolysis increased in the early timepoints after addition of H_2O_2 (15 and 30 min) and declined rapidly at the later timepoints. Triosephosphate isomerase (*TPI1*), glyceraldehyde-3-phosphate dehydrogenase (*TDH3)* and glyceraldehyde 3-phosphate dehydrogenase (*TDH2)* genes were up-regulated in the presence of melatonin during the stress period. The chemoautrophic energy pathway class

had the highest fold change. However, the only gene differentially expressed in this class was the formate dehydrogenase 1 *(FDH1)*. This gene catalyzes the NAD+-dependent oxidation of formate to carbon dioxide which is the final step in the methanol oxidation pathway in methylotrophic microorganisms (Sakai et al., 1997). On a molecular function level, it also has oxidoreductase activity (Fig 4.12A).

The expression of genes involved in fatty acids and lipids, amines and polyamines, secondary metabolites and cofactors, prosthetic groups, electron carriers' metabolic pathways increased in the first 30 min after addition of H_2O_2 and decreased thereafter. (Fig 4.12C). In the fatty acids and lipids category, genes associated with sterol and long chain fatty acid biosynthesis were up-regulated. In the amines and polyamines category, expression of choline-phosphate cytidylyltransferase (*PCT1*), phospholipase D (*SPO14*), sn-1,2-diacylglycerol ethanolamine- and cholinephosphotranferase (*EPT1*) and spermidine synthase (*SPE3*) was increased in the presence melatonin. Trehalose biosynthesis genes TSL1 (trehalose synthase complex regulatory subunit) and *TPS1* (trehalose-6-phosphate synthase) were up-regulated in the metabolic regulator's category. Cellular processes, central dogma and regulation pathways were unaffected by treatments in this study (Fig 4.12B).

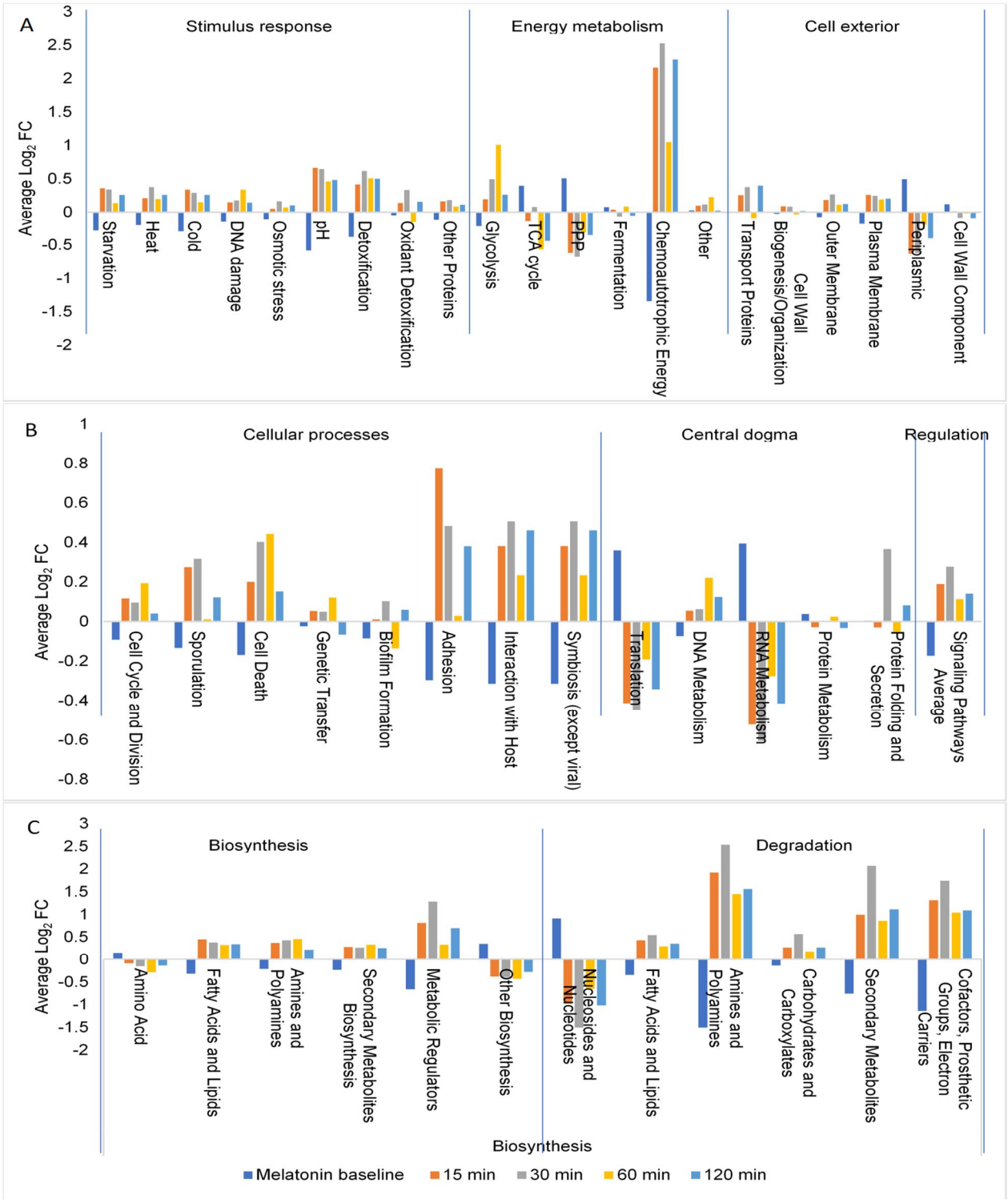

Figure 4.12: Impact of melatonin (300 µM) on the transcriptional response of *S. cerevisiae* at baseline and during H_2O_2 induced oxidative stress in nitrogen-limited continuous culture

4.5 Discussion

Oxidative stress caused by accumulation of reactive oxygen species (ROS) is a naturally occurring biological process that occurs in all respiring cells; however this can cause damage to proteins, DNA and lipids resulting in impaired cell function (Sha et al., 2013). Cellular response of yeast to oxidative stress induced by agents such as H_2O_2, various hydroperoxides, superoxide anions and heavy metals has been studied extensively (Gasch et al., 2000; Causton et al., 2001; Sha et al., 2013; Zhao et al., 2015); however these perturbation studies were performed in batch cultures. Possible limitations of conducting perturbation experiments in batch culture systems is the changing physico-chemical conditions which could increase the complexity of the datasets. On the other hand, continuous culture systems provide a constant environmental milieu and remove growth effects that could mask subtle physiological changes (Hoskisson and Hobbs., 2005). To the best of our knowledge, this is the first study utilising a nitrogen-limited continuous culture system to investigate the physiological and transcriptional response of steady state cultures of *S. cerevisiae* to the indoleamine melatonin, pre- and post-H_2O_2 induced oxidative stress. The experimental set-up allowed us to maintain cells in exponential growth phase and monitor the recovery process at genomic, metabolic and physiological level.

Melatonin is a metabolite with a plethora of biological functions in higher and lower organisms. Majority of the studies characterising the biosynthesis and functions of melatonin were performed on vertebrates and plants, but its metabolism is less understood. Metabolism of melatonin occurs enzymatically, or via interaction with ROS and nitrogen species resulting in formation of a variety of metabolites including cyclic 3-hydroxymelatonin, 6-hydroxymelatonin and *N*-acetyl-*N*-formyl-5-methoxyknuramine (Tan et al., 2007). However, there is very little information on the metabolism of melatonin in microorganisms or the functions of these metabolites. The synthesis of melatonin is inducible under moderate oxidative conditions in animals, plants and microalga and once formed, it is rapidly metabolised (Tan et al., 2015). This production pattern, albeit at very low concentrations and excreted into the medium was observed in this study (Table 4.3) where the endogenous levels of melatonin decreased after addition of H_2O_2. The addition of exogenous melatonin allowed us to quantify and monitor the production patterns of 6-hydroxymelatonin. This hepatic metabolite and photodegradation product of melatonin has a higher antioxidant efficiency when compared to melatonin (Álvarez-Diduk et al., 2015). In this study the relative activity of 6-hydroxymelatonin was 19 times faster than melatonin in non-polar solution and four times faster in aqueous solutions. In the current study levels of 6-hydroxymelatonin increased rapidly soon after addition of H_2O_2 and then decreased rapidly whereas the levels of melatonin

remained relatively stable. The data suggests that once produced, 6-hydroxymelatonin could compliment melatonin in the detoxification of ROS in yeast but this needs further investigation. To the best of our knowledge, this is the first report detailing the production of this metabolite in yeast.

The OSR is largely regulated at a transcriptional level and different ROS have been shown to elicit distinct transcriptional responses (Jamieson, 1998). In a general response to oxidative stress in yeast, transcriptional factors (*YAP1, SKN7* and *MSN2/4*) regulate the expression profile of genes to return cells to redox homeostasis. One of the cellular antioxidants regulated by *YAP1* transcription factor in response to accumulation of ROS is glutathione (GSH) and it is thought to be the most abundant redox scavenging molecule in cells (Marchler et al., 1993). Glutathione is synthesized in a two step process by the enzymes γ-glutamylcysteine synthetase (*GSH1*) and glutathione synthetase (*GSH2*) (Lisowsky, 1993; Grant et al., 1997). During oxidative stress, it neutralises ROS by donating electrons to them and is oxidized to form GSSG (Farrugia and Balzan, 2012). Thus, the presence of ROS results in a decrease in GSH and an increase in GSSG. Enzymes involved in the maintenance of the GSH/GSSG redox balance are glutathione reductase (*GLR1*) which reduces GSSG to GSH in an NADPH-dependent process; glutathione peroxidase, which reduces H_2O_2 by oxidizing GSH to GSSG; and glutaredoxins, which regulate the protein redox state using GSH and NADPH (Morano et al., 2012).These genes were up-regulated in this study in response to addition of H_2O_2 (Fig 4.10, 4.11 and 4.12).

Our transcriptomic data revealed that the melatonin supplementation buffered the genomic response to oxidative stress. Prior to induction of stress, genes involved in sulphur metabolism (*MET28, MET1, MET2, MET14, MET16* and *MET17*) were over-expressed in melatonin-treated cells. The induction of sulphur metabolism in the context of oxidative stress results in the formation of cysteine which is component of molecules such as glutathione, glutaredoxin, thioredoxin and Yap1p (Sha et al., 2013). This over-expression in unstressed yeast cells could have primed the cultures to respond to subsequent oxidative stress more efficiently. The temporal response of *S. cerevisiae* to H_2O_2 in this study separated into early (15 and 30 min) and late (60 and 120 min) response. The gene expression profile in the early response showed an induction of oxidative stress responsive genes associated removal of ROS. This induction decreased over time and were near baseline levels at 120 min. Similar expression profiles were described in stress perturbation experiments performed in batch cultures (Gasch et al., 2000). The potential priming effect of melatonin mentioned above was observed in the increased expression of transcription factors *MSN2/4* and glutathione system related genes (*GRX6, GPX2, GLR1* and *YCF1*). The monothiol glutaredoxin *GRX6* is found in the early secretory pathway and is act in sulfhydryl regulation during stress conditions. *GPX2*

protects membrane lipids against peroxidation whereas *YCF1* transports GSSG that is not reduced immediately to the vacuole. Recently two studies have described the expression profile of some antioxidant genes in response to H_2O_2 stress in the presence of melatonin in yeast. Vazquez et al (2017) found that melatonin increased the expression of *GSH1, GPX1, ZWF1, GLR1, CTT1, CTA1, SOD1, SOD2* and *TRX2* 120 min after the addition of 2 mM H_2O_2 to *S. cerevisiae* cells in exponential growth. Although this study described the influence of melatonin on glutathione related genes 45 min after stress, our data suggests that this response occurs much sooner in cells (within 30 min), but this observation requires further investigation. Bisquert et al (2018) investigated the short transcriptional response of *CTT1, GPX2, SOD1, TRX2, CTA1, GPX3, SOD2* and *TRX3* to H_2O_2 stress in presence and absence of melatonin. Except for *TRX2*, melatonin did not have a strong impact on the expression of these genes. However, we found that the expression levels of *GPX2* were higher in the melatonin treated cells 30 min after induction of oxidative stress. Our data also shows for the first time the impact of melatonin on the expression of transcription factors in yeast.

Global responses of yeast to environmental stressors encompasses a larger stress signalling network that integrates information from several pathways, thereby ensuring survival of yeast. Trehalose has been shown to protect yeast cells against oxidative stress by quenching ROS and acting as a membrane protectant (Benaroudj et al., 2001). In this study, trehalose-6-phosphate synthase (*TPS1*) which is involved in the control of glycolytic flux was up regulated in the early response phase and then down-regulated as cells recovered from stress. A similar expression profile was observed with the polyamines choline-phosphate cytidylyltransferase (*PCT1*), phospholipase D (*SPO14*), sn-1,2-diacylglycerol ethanolamine- and cholinephosphotranferase (*EPT1*) and spermidine synthase (*SPE3*) and there is some evidence that polyamines confer some protection to yeast against oxidative (Jamieson, 1998). Sterols are essential parts of all cells and the predominant sterol in *S. cerevisiae* is ergosterol. Sterols play an important role in maintaining the fluidity and permeability of membranes (Shobayashi *et al.*, 2005). When cells are stressed, sterol and fatty acid biosynthesis plays a role in the maintenance of membrane efficiency. Oleic acid and ergosterol supplementation have been found to mitigate oxidative stress in wine strains of *S. cerevisiae* (Landolfo et al., 2010). In our data the expression of several genes in the ergosterol biosynthetic pathway were up-regulated in the melatonin treatment 15 min after the induction of stress (Fig 4.9).

In conclusion, this study suggests that the activity of melatonin in *S. cerevisiae* during oxidative stress is not targeted at a single pathway. It utilises a multipronged approach to reduce ROS accumulation, repair cell damage and increase recovery rate from stress. As free radical scavengers and antioxidants, melatonin and 6-hydroxymelatonin could have increased the rate ROS removal. At a genomic level melatonin seems to have primed the cells to activate

OSR genes through the induction of sulphur metabolism which lead to the over expression of glutathione and glutaredoxin genes but these observation needs to be investigated further. This data could highlight the transcriptomic importance of melatonin in cell membrane repair after oxidative damage.

4.6 Supplementary information

Figure S4.1: Principle component analysis showing separation of transcriptomic data

Figure S4.2: Top 40 of the genes differently expressed in *S. cerevisiae* treated with 300 µM melatonin

Table S4.5: Total number of mapped reads per biological repeat in each sampling/treatment group when aligned to the *S. cerevisiae* S288C genome

Table S4.2: Genes differentially expressed by melatonin treatment at baseline (A) and biological processes enriched by upregulated genes (B) and down regulated genes (C)

Table S4.3: Comparison of up-regulated genes at 15, 30, 60 and 120 min and melatonin baseline derived from Venn diagram (A&B) and GO processes enriched by the genes (C&D)

Table S4.4: Comparison of down-regulated genes at 15, 30, 60 and 120 min and melatonin baseline derived from Venn diagram (A&B) and GO processes enriched by the genes (C&D)

Table S4.5: Biological processes enriched by genes differentially expressed at 15 and 30 minute timepoints and REVIGO ranking of these processes in melatonin + H_2O_2 treatment at 15 min (A &B) or 30 min (C&D) and H_2O_2 only treatment at 15 min (E&F) or 30 min (G&H).

Table S4.6: Biological processes enriched by genes differentially expressed at 60 and 120 minute timepoints and REVIGO ranking of these processes in melatonin + H_2O_2 treatment at 60 min (A &B) or 120 min (C&D) and H_2O_2 only treatment at 60 min (E&F) or 120 min (G&H).

Table S4.7: Biological process enriched and REVIGO ranking of down-regulated genes differentially expressed in the presence and absence of melatonin at 15 minutes (A&B), 30 minutes (C&D), 60 minutes (E&F) and 120 min (G&H)

Table S4.8: Genes differentially expressed by melatonin with and without H_2O_2 stress (A) and differentially expressed by H_2O_2 without melatonin (B)

Table S4.9: Biosynthesis (A), Cell exterior (B), Cellular Processes (C), Central dogma (D), Degradation (E), Energy metabolism (F), Regulation (G) and Stimulus response (H) pathways enriched by melatonin at baseline and during H_2O_2 induced oxidative stress

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Chapter 5

Impact of melatonin supplementation on yeast stress response

Chapter 5

Impact of melatonin supplementation on yeast stress responses

5.1 Abstract

Yeast utilise various mechanisms to detoxify reactive oxygen species and repair damage to cellular structures. Antioxidants such as melatonin form part of this arsenal and the ROS scavenging properties of this indoleamine are well studied. Although studies on the physiological functions of melatonin in yeast are increasing, data generated thus far are limited to response against the oxidative stress agent, hydrogen peroxide. However, yeast cultures are exposed to various stressors when growing in different environments. This study assessed the impact of melatonin against oxidative (H_2O_2, menadione), reductive (dithiothreitol), acid (acetate), heavy metal ($CuCl_2$), osmotic (NaCl) and thermal stress in *S. cerevisiae* and *Kazachstania* yeast strains. Cultures were spotted on medium containing stress-inducing compounds with or without 300 µM melatonin. In addition to this, the expression of some genes involved in oxidative stress response as well as ergosterol biosynthetic pathways and sulphate assimilation pathways in a wild type *S. cerevisiae* strain in the presence and absence of melatonin (300 µM) and stress (H_2O_2, menadione and dithiothreitol) was evaluated by qPCR. Melatonin improved the survival of *S. cerevisiae* wild type and deletion mutant strains in H_2O_2, menadione, dithiothreitol and thermal stress plate assays as well as *Kazachstania* strains in H_2O_2 stress plate assays. This protective effect on *S. cerevisiae* strains was not observed in the acetate, NaCl and $CuCl_2$ assays. Gene expression analysis showed that melatonin modulated the expression of *MET* genes in the unstressed cells and of antioxidant genes after the addition of stressors. The data reveals that the protective effects of melatonin in yeast is not limited to H_2O_2 stress. It also suggests that melatonin might prime yeast cultures to respond to subsequent stress more efficiently by inducing the expression of sulphur metabolism related genes. Once stress is induced, the expression of some antioxidant genes is altered resulting in faster recovery of cells from the stress. Therefore, melatonin may act as a general antioxidant in yeast but there is no apparent direct physiological role that can be attributed to this indoleamine.

Key words: Melatonin, *Saccharomyces cerevisiae*, abiotic stress, gene expression

5.2 Introduction

The use of oxygen to extract energy from food sources has both beneficial and detrimental impacts on living organisms (Benzie, 2000). Aerobic metabolism generates oxygen radicals and other activated oxygen species which cause oxidative stress (Davies and Percy, 2000). If uncontrolled, oxidative stress can cause damage to lipids, carbohydrates, proteins and nucleic acids, potentially leading to cell death (Moradas-Ferreira and Costa, 2000). Cells are equipped with regulatory transcription factors that control the expression of oxidant-inducible gene in response to oxidative stress. These transcription factors include *YAP1, CUP1, HAP1, MSN2, MSN4* and *SKN7* which regulate the expression of antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase, glutaredoxin and thioredoxins to protect cells from oxidative damage by reacting with reactive oxygen species (ROS) (Moradas-Ferreira et al., 1996; Jamieson, 1998). In addition to enzymatic machinery, organisms produce or take up small antioxidant molecules from the environment such as glutathione (GSH), ascorbic acid, tocopherol and melatonin (MEL), which delay or prevent oxidative damage (Amari et al., 2008).

MEL is a ubiquitous indoleamine with numerous physiological functions in various organisms. Its most notable function in vertebrates is the regulation of circadian rhythms, but it also has an impact on seasonal reproduction, immunomodulation and ageing (Pandi-Perumal et al., 2006). In plants, MEL was shown to promote growth (Murch and Saxena, 2002; Hernández-Ruiz et al., 2005; Hernández-Ruiz and Arnao, 2008; Chen et al., 2009; Arnao, 2014) as well as increasing photosynthetic efficiency and decreasing the rate of chlorophyll degradation in senescent leaves (Arnao, 2014). In response to thermal, desiccation, osmotic and heavy metal stresses, plants increase production of MEL (Hardeland, 2016; Shi et al., 2016). As an antioxidant MEL directly scavenges ROS (Tan et al., 2015) and binds several toxic metals (Romero et al., 2014), reducing the damage caused. It also stimulates the production of GSH (Rodriguez et al., 2004), activates antioxidant enzymatic defence systems (Yu et al., 2018), and improves the efficiency of the mitochondrial electron transport chain (Acuna-Castroviejo et al., 2007; López et al., 2009) resulting in reduced free radical formation.

Research on the physiological role of MEL in yeast is still in its infancy and the activities of MEL in these organisms still requires investigation. Recently Vazquez et al. (2017) and Bisquert et al. (2018) focused on H_2O_2 induced stress and found that MEL enhanced the expression of some antioxidant genes (*CTT1, CTA1, GPX1, GPX2, GPX3, GLR1, SOD1, SOD2*, *TRX2, TRX3 and ZWF1*) in *S. cerevisiae* in response to oxidative stress. MEL was also found to lower ROS accumulation and lipid peroxidation while modulating the fatty acid composition of both *S. cerevisiae* and non-*Saccharomyces* yeasts exposed to H_2O_2 stress

(Vázquez et al., 2018). The results reported in Chapter 4 identified several additional genes that were up-regulated by MEL pre- and post- H_2O_2 stress including *MET2, MET14, MET16, MET17, MET28, GRX6, GPX2, GLR1*, *YCF1* and transcription factors *MSN2/4*.

The aim of the present study was to assess if the effectiveness of MEL as an antioxidant in yeast is dependent on the oxidative stress agent employed, and whether other forms of stress elicit MEL-dependent responses. For this purpose, *S. cerevisiae* wild type, lab strains and deletion mutants as well as several *Kazachstania* strains were exposed to oxidative, reductive, heavy metal, osmotic and thermal stress in the presence and absence of 300 µM MEL. *Kazachstania* strains were included in this present study because they produced the highest levels of MEL in SGJ (Chapter 3). In addition, the expression of genes of the oxidative stress response (*SOD2*, *GPX1*, *GPX2*, *GLR1*, *GRX3*, *GRX6)*, and genes of the ergosterol biosynthetic (ERG4 and ERG 26) and sulphate assimilation (*MET2, MET14, MET16, MET17, MET28*) pathways was evaluated in a wild type *S. cerevisiae* strain in the presence and absence of MEL and stress agents (H_2O_2, menadione and dithiothreitol (DTT)). Finally, the levels of MEL and 6-hydroxymelatonin were quantified.

5.3 Materials and methods

5.3.1 Yeast strains and growth conditions

The wild type strain *S. cerevisiae* BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) and all single gene knock-outs (*Δgpx1, Δgpx2, Δsod2, Δglr1, Δgrx3, Δerg4, Δtrx2, Δyap1, Δmsn2, Δmsn4 and Δhsp104)* used in this study were obtained from Euroscarf (Frankfurt, Germany). *S. cerevisiae* IWBT Y805, *Kazachstania aerobia* IWBT Y845 and IWBT Y895 and *Kazachstania servazzii* IWBT Y0235, IWBT Y0178 were obtained from the culture collection of the South African Grape and Wine Research Institute at Stellenbosch University. *K. aerobia* CBS 9918 *and K. servazzii* CBS 4311 were obtained from the Westerdijk Fungal Biodiversity Institute (CBS) collection. Cultures were grown in minimal medium (MM) containing 6.7 g/L yeast nitrogen base (YNB) with amino acids (DIFCO) and 20 g/L glucose supplemented with amino acids according to the specific requirements of the respective strains.

5.3.2 Stress assays

The plates used for the assessment of hydrogen peroxide (H_2O_2), menadione, dithiothreitol (DTT), acetic acid, cupric chloride ($CuCl_2$) and sodium chloride (NaCl) in combination with MEL were prepared the day before cultures were spotted. MM agar medium was left to cool to 50°C before the addition of the stressors to a final concentration of 0.9, 1, 1.2 and 1.6 mM H_2O_2; 0.025 and 0.035 mM menadione, 6 and 9 mM DTT, 0.75 mM $CuCl_2$, 1.2 M NaCl and

0.4% acetic acid. MEL was added to a final concentration of 300 µM. For the evaluation of stress tolerance on plate assays, overnight pre-cultures of cells grown in MM were inoculated into fresh MM and grown to OD_{600} of ± 1. Five 1:10 serial dilutions were made and 10µL droplets of each dilution were spotted onto MM agar containing stressors in the presence and absence of an agar concentration of 300 µM MEL. The plates were incubated at 30°C for three days after which growth was monitored.

Cultures used for thermal stress experiments were grown as described above. MEL treated cultures were treated with a final concentration of 300 µM MEL for 60 min before the induction of stress. Cells were harvested, washed once and resuspended in sterile saline. Aliquots of 1 mL were dispensed into 1.5 mL microcentrifuge tubes and incubated at 50°C for 15 and 30 min in a water bath. Five 1:10 serial dilutions were made and 10µL droplets of each dilution were spotted onto MM agar. The plates were incubated at 30°C for three days after which growth was monitored.

5.3.3 Transcriptional response analysis

Cells precultured in MM overnight were reinoculated into fresh 20 mL volumes of MM and grown to OD_{600} of 0.6. MEL treated cultures were treated with 300 µM MEL for 60 min before the induction of stress. Sublethal concentrations of H_2O_2 (0.6 mM), menadione (0.1 mM) and DTT (1 mM) were added to MEL treated and untreated cultures. Samples were taken before the addition of stress and 5, 15 and 30 min after addition of stress for RNA extraction. Yeast viability after exposure to stress (stressor with MEL or stressor alone) in comparison with cells without stress (MEL or control) was evaluated by a microplate bioassay in which 96-well plates were prepared by dispensing 250µL of YNB broth inoculated with cells of each condition into each well to obtain an initial OD_{600} of 0.050. The microplate was incubated at 28°C for 24 h, and OD_{600} was measured every 30-min using a microplate reader. Experiments were performed in triplicate.

5.3.4 RNA extraction and reverse transcription

RNA was extracted as previously described with modifications (Collart & Oliviero, 1993). Briefly, the cells were harvested from 20 mL samples taken before and after the addition of stress by centrifuging for 5 min at 8500 rpm, washed and snap-frozen in liquid nitrogen. All samples were stored at -80°C until RNA extraction. Cells were lysed with a high salt buffer (0.5 M NaCl, 20 mM Tris/HCl, 10 mM EDTA and 2% SDS) and 100 µL acid washed beads. Cells suspension was vortexed for 3 min and then centrifuged for 5 min at 20000 X g. The pellet was re-suspended in the solution and 200 µL of acid phenol and chloroform was added. Cell suspensions were vortexed for 1 min and centrifuged for 10 min at 15000 X g. The

aqueous layer was transferred to a new 1.5 mL reaction tube and 400 µL of chloroform was added. After shaking for a few seconds, the reaction tubes were centrifuged for 10 min at 15000 X g. The aqueous layer was transferred to a new reaction tube and 1 mL 100% ice cold isopropanol was added. RNA solution was stored at -20°C overnight to allow for precipitation. The reaction tubes were centrifuged at maximum speed for 10 min at 4°C and the supernatant was discarded. Pellet was dried in a laminar flow cabinet and then resuspended in 50 µL diethyl pyrocarbonate (DEPC) treated distilled water. Samples were DNase treated (Promega RQ1 RNAse free DNase) to eliminate contamination with genomic DNA. The RNA was re-precipitated overnight at -20°C with 0.1 volume of 3 M sodium acetate (pH 5.2), 2.5 volumes of 96-100% ethanol to one volume of sample. The reaction tubes were centrifuged at maximum speed for 10 min at 4°C and the supernatant was discarded. Pellet was dried in a laminar flow cabinet and then resuspended in 20 µL DEPC treated dH_2O. The concentration and purity of the extracted RNA samples were established using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNA integrity and the absence of chromosomal DNA were determined by gel electrophoresis (1.2% agarose gel, 1 h at 100V). RNA PCR was performed to confirm the absence of genomic DNA and PCR products were run on 1% GelRed® (Biotium, Fremont, CA, USA) stained agarose gel. DNase treated RNA was diluted to 180 ng/µL and used for cDNA synthesis. The cDNA was synthesised using the ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA) with $Oligo(dT)_{15}$ primers.

5.3.5 Quantitative real time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qPCR) was performed on an Applied Biosystems 7500 Real-Time PCR System with 7500 Software version 2.3 (Life Technologies, United States) using the Ampliqon RealQ plus 2x Master Mix Green (Ampliqon A/S, Denmark) for gene detection. The qPCR reaction was set up as proposed by the manufacturer. The housekeeping genes were *ACT1* and *UBC6* and primers used to determine transcript levels in this study are listed in Table 5.1. A negative control was included in each run. Thermal cycling conditions for amplification were as follows: initial denaturation at 95°C for 15 min, followed by 35 cycles of 95°C for 15 s, 58°C for 30 s and 72°C for 32 s. Fluorescence data were collected during each elongation step. The specificity of the qPCR for each primer pair was verified by a melting curve, which was established by an additional step starting from 95°C for 15 s to 58°C for 1 min. Serial dilutions of cDNA were used to determine the qPCR efficiency (*E*) for each primer set which was calculated using the formula $E = 100 \times (10^{-1/slope} - 1)$.

Table 5.1: Primers used in this study

Gene description	Primer sequences (5′ to 3′)	Amplicon size (bp)
SOD2	F: CAGTCTGGACGAGCTGATTAAG R: CTTGCCTCCATTAGAGAGGTTT	100
GPX1	F: GCAAGATCCCGTCTACAAGTT R: CACCTTCCCATTTCGGTCT	109
GPX2	F: CTTCACGCCGCAGTATAAAGA R: CCTGCTTCCCGAACTGATTAC	101
GLR1	F: TGTGCGGGACCAAATGAA R: CCTTAGTGGCACCCATCTTT	106
GRX3	F: GACGACGAAGAAGAGGAAGAAG R: ACCCGCATTTAGGTTCAGAG	121
GRX6	F: GCGAACTTCTTGACGGTAGAG R: AAGTCGGCATAGCAGCATATT	109
ERG4	F: TGGAGAACGGTATCCCAGAA R: AAATCCCGGGCAACGTATAG	93
ERG26	F: GTGGAAGGCAGATGGTCATATT R: GGCTCTTTACCCAGCATCTT	111
MET2	F: CGGGTGAAGTGAAGGACATAA R: GGCGTCGAACCTATTGATGA	98
MET14	F: GGAGGGTGTAATCAAGGAGTTT R: TTCTTCAACCGTCTTCTGGTC	100
MET16	F: GGACGTTCGAGCAGGTTAAA R: CGGGTTGTGTGGAATGGTAA	108
MET17	F: CCCTGGTTTAGCATCTCATTCT R: CAGTTTCCTTGTCGGCATTTG	116
MET28	F: GCAAGCAGGAAGGGTATGAA R: TGCTAACCAGCAGCAATGA	109
ACT1	F: CGTGCTGTCTTCCCATCTATC R: CATCACCAACGTAGGAGTCTTT	97
UBC6	F: GCAGGCTCACAAGAGATTGA R: CCGCAGGTCCTGTGATAATATAG	125

The relative expression (RE) was calculated from the threshold cycle value (C_T) data using the comparative critical threshold ($\Delta\Delta C_T$) method (Livak and Schmittgen, 2001). The following equations were used to determine the relative expression:

The ΔC_T value for each sample was determined by calculating the difference between the C_T value of the target gene and the C_T value of the endogenous housekeeping gene. This was determined for each treated sample as well as for the control sample.

$$\Delta C_T = C_T \text{ of target gene} - C_T \text{ of housekeeping gene}$$

$$\Delta\Delta C_T = \Delta C_T \text{ of treated condition} - \Delta C_T \text{ of control condition}$$

The normalized level of target gene expression is calculated by using the formula:

$$RE = 2^{-\Delta\Delta CT}$$

The treated condition was the MEL treated samples 0 min (before addition of stress) and 5, 15 and 30 min after addition of stress. Control conditions were the MEL untreated samples at

the corresponding timepoints. Three biological replicates were analysed for each timepoint and each condition. Data were subjected to one-way analysis of variance (ANOVA) and Tukey's *post hoc* test to evaluate the effect of each treatment. The results were considered significant at a *p*-value less than 0.05 (XLSTAT Software).

5.3.6 Metabolite analysis

Supernatant samples from liquid assays were analysed for presence and quantification of MEL and 6-hydroxymelatonin. External standards for these metabolites were purchased from Sigma-Aldrich (St. Louis, MO, USA). The LC-MS/MS analysis was carried out on an Acquity UPLC separation module (Waters, Milford, MA, USA) coupled with a Xevo TQ MS (Waters) triple quadrupole mass spectrometer. A Waters BEH C 18 column (100 x 2.1 mm, 1.7 µm) was used for separation. The mobile phase solvents were 0.1% formic acid (A) and acetonitrile (B). Separation was performed in gradient mode using the following elution profile: 5–60% (A in B) for six min and then 60–100% (A in B) for three min at a flowrate of 0.45 mL/min. The column and samples were kept at 40 and 20°C, respectively. The capillary voltage was set to 3.0 kV, the cone voltage was 14 V, the source temperature was 130°C, the desolvating temperature was 350°C and the desolvation gas (nitrogen) was at 800 L/h. Argon was used as collision gas. MEL was determined in multiple reaction monitoring (MRM) mode, and the data were acquired by MassLynx 4.0 software (Waters). The mass spectrometer operated in the electrospray ionization (ESI) positive mode and the fragmentation transitions are described in Table 3.2. To keep the cone clean, the eluate from the first 1.1 min analysis was discarded by the divert valve.

5.4 Results

5.4.1 The impact of melatonin on environmental stresses

The role of MEL in protecting *S. cerevisiae* and *Kazachstania* yeast strains against various environmental stressors was evaluated on plate assays (Figs 5.1 and 5.2). MEL supplementation alone did not have an impact on the growth of the yeast strains. However, it improved the growth of *S. cerevisiae* BY4742 and IWBT Y805 cultures with 0.025 mM menadione and 9 mM DTT and H_2O_2 stress (Fig 5.1). MEL did not improve the growth of these cultures in plates containing NaCl and acetic acid (data not shown).

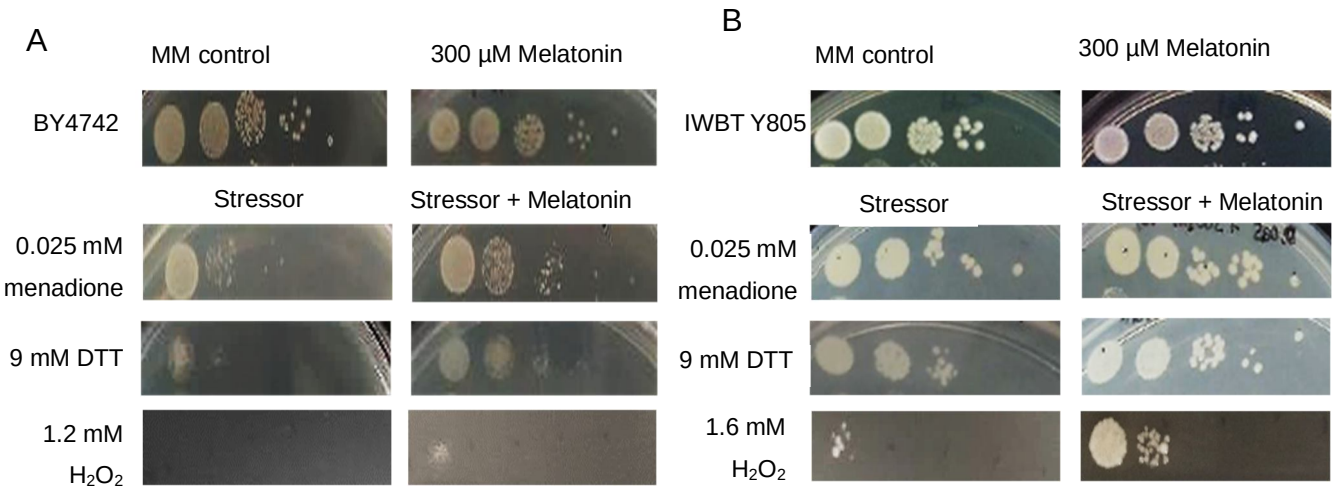

Figure 5.1: The effect of melatonin supplementation on growth of *S. cerevisiae* lab strain BY4742 (A) and IWBT Y805 (B) spotted on MM agar containing various stress agents. Ten microliters of 10-fold serial dilutions were spotted on MM plates with and without melatonin.

The concentration of H_2O_2 (0.75 mM) used to assess the tolerance of *Kazachstania* strains to oxidative stress elicited a very strong response which inhibited the growth of most of the strains except for *K. servazzii* IWBT Y0235 in the absence of MEL. However, MEL supplementation improved the tolerance of all strains to H_2O_2 stress in a concentration dependent manner. (Fig. 5.2). At a concentration of 0.75 mM H_2O_2 in combination with 0.1 mM of MEL all strains displayed improved H_2O_2 tolerance. However, only three strains (*K. aerobia* IWBT Y845, *K. aerobia* CBS 9918 and *K. servazzii* IWBT Y0235) showed improved growth when medium was supplemented with lower levels of MEL (0.01 mM) whereas the growth of *K. aerobia* IWBT Y845, *K. aerobia* IWBT Y895, *K. aerobia* CBS 9918 and *K. servazzii* IWBT Y0235 was improved in plates supplemented with higher concentration of MEL (1 mM).

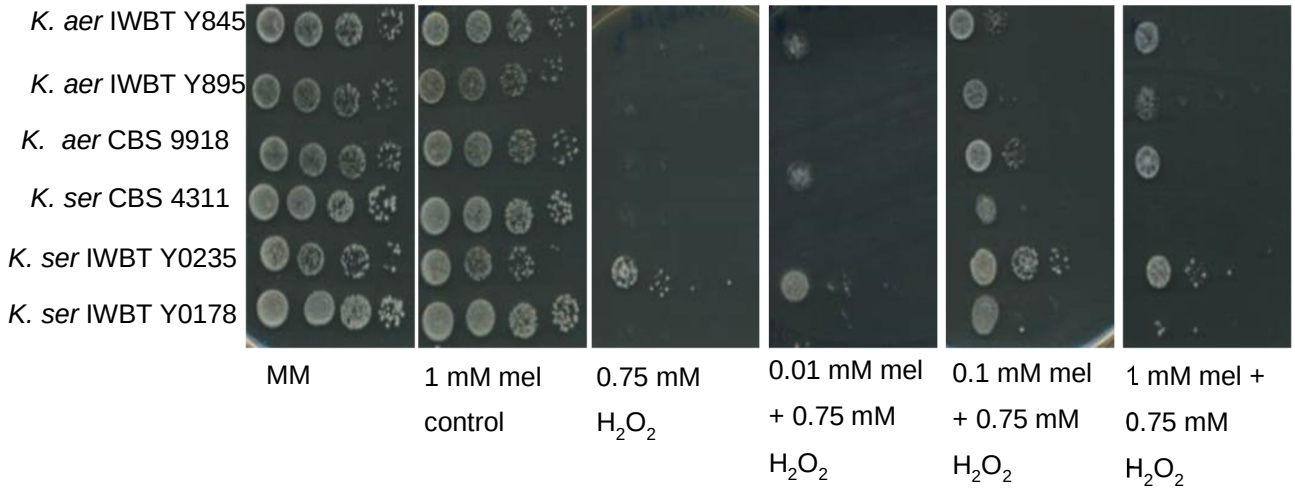

Figure 5.2: The effect of melatonin supplementation on growth of *Kazachstania aerobia* (IWBT Y845, IWBT Y895 and CBS 9918) and *Kazachstania servazzii* (IWBT Y0235, IWBT Y0178 and CBS 4311) strains spotted on MM agar containing 0.75 mM H_2O_2 and various concentrations of melatonin. Ten microliters of 10-fold serial dilutions were spotted on MM plates with and without melatonin.

5.4.2 Impact of melatonin on yeast deletion strains

Several studies here (Chapter 4) and elsewhere (Vázquez et al., 2017; Bisquert et al., 2018) have shown that MEL up-regulates antioxidant related genes during H_2O_2 induced oxidative stress. To assess if the action of melatonin is related to genes highlighted previously in the transcriptomic analysis (Chapter 4), strains bearing deletions of genes involved in antioxidant response and ergosterol biosynthesis were assessed for their response to stress imposed by oxidative (0.9 and 1 mM H_2O_2; 0.035 mM menadione), reductive (6 mM DTT), osmotic (1.2 NaCl), heavy metal (0.75 mM $CuCl_2$) and thermal (50 °C for 15 and 30 min) stresses in the presence or absence of 300 µM MEL. MEL supplementation enhanced the survival of Δ*gpx1*, Δ*gpx2, Δglr1, Δgrx3, Δmsn2, Δmsn4 and* Δ*erg4* strains when exposed to 0.035 mM menadione (Fig 5.3 B). Menadione at the concentration used in this study completely inhibited the growth of Δ*sod2* and Δ*yap1* and MEL supplementation had no impact on the survival of these strains as well as Δ*trx2*. When cultures were exposed to DTT stress (Fig 5.3 C), MEL improved the survival of all the deletions strains except for *Δyap1* where growth was completely inhibited. The 0.9 mM concentration of H_2O_2 completely inhibited the growth of *Δyap1* and impaired the growth of *Δglr* and *Δtrx2* (Fig 5.3D) but it had very little impact on the growth of the other deletion mutants (data not shown). When the concentration was increased to 1 mM, H_2O_2 reduced the growth of all strains. MEL supplementation enhanced the survival of Δ*gpx1*, Δ*gpx2, Δglr1,* and *Δmsn4* but did not improve the survival of the remaining mutants. The inhibition of the *Yap1* mutant by the reductive and oxidative agents in this study is to be expected as this transcription factor is critical for the tolerance to oxidative agents (Morano et

al., 2012). The impact of MEL on growth of mutants did not suggest that MEL acts through any specific stress responsive pathway suggesting that the impact is due to the intrinsic antioxidant activity of the compound and not linked to any specific genetic interaction or regulation.

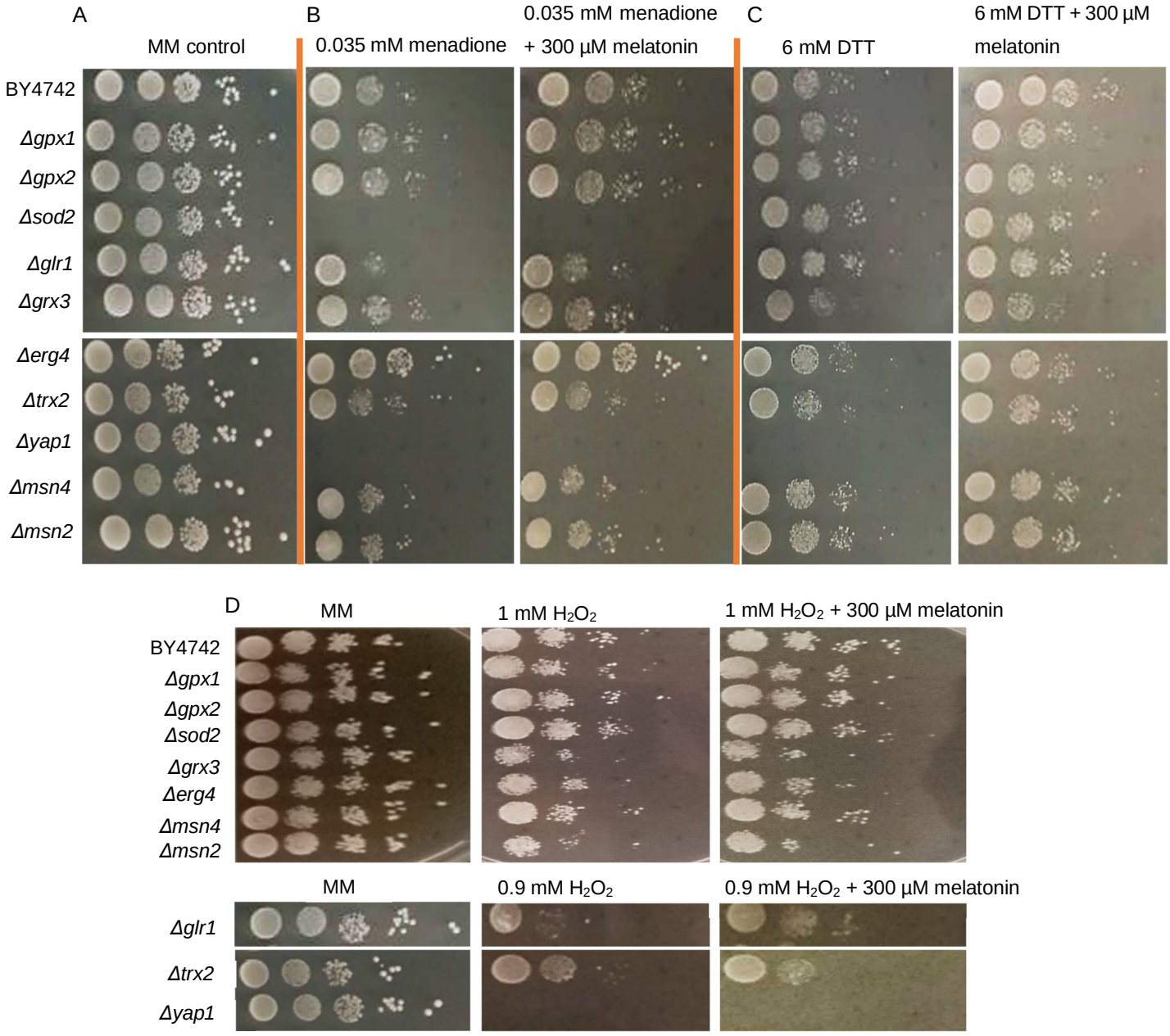

Figure 5.3: The effect of melatonin supplementation on growth of *S. cerevisiae* lab strain BY4742 and single gene deletion mutants of genes required for the cells defence against oxidative (B and D) and redox (C) stress. Ten microliters of five 10-fold serial dilutions were spotted MM agar only (A) or MM containing menadione (B), DTT(C) and H_2O_2 (D) with and without 300 µM MEL.

MEL treated and untreated cultures were exposed to thermal shock (Fig 5.4). A heat shock mutant was included in the experiment as a control to ensure that the heat shock response was observed. Although 15 min thermal shock affected the survival of *Δhsp104* (Fig 5.4 B), it did not have a strong enough impact on the other cultures to assess the impact of MEL. Incubation of the cultures for 30 min elicited a stronger response (Fig 5.4C). MEL supplementation did not have an impact on the growth of BY4742 and the *Δgpx1*, *Δsod2*, *Δmsn4* and *Δhsp* deletion mutants. However, this indoleamine seemed to enhance the survival of *Δglr1, Δgrx3, Δtrx2, Δerg4 and Δmsn2* strains. When cultures were spotted on plates containing $CuCl_2$ and NaCl, the concentration of $CuCl_2$ used in this study completely inhibited the growth of *Δgrx3* and the growth of *Δerg4* was strongly impacted by both $CuCl_2$ and NaCl. However, MEL did not have an impact on the survival of any of the cultures to these stressors (Fig 5.5).

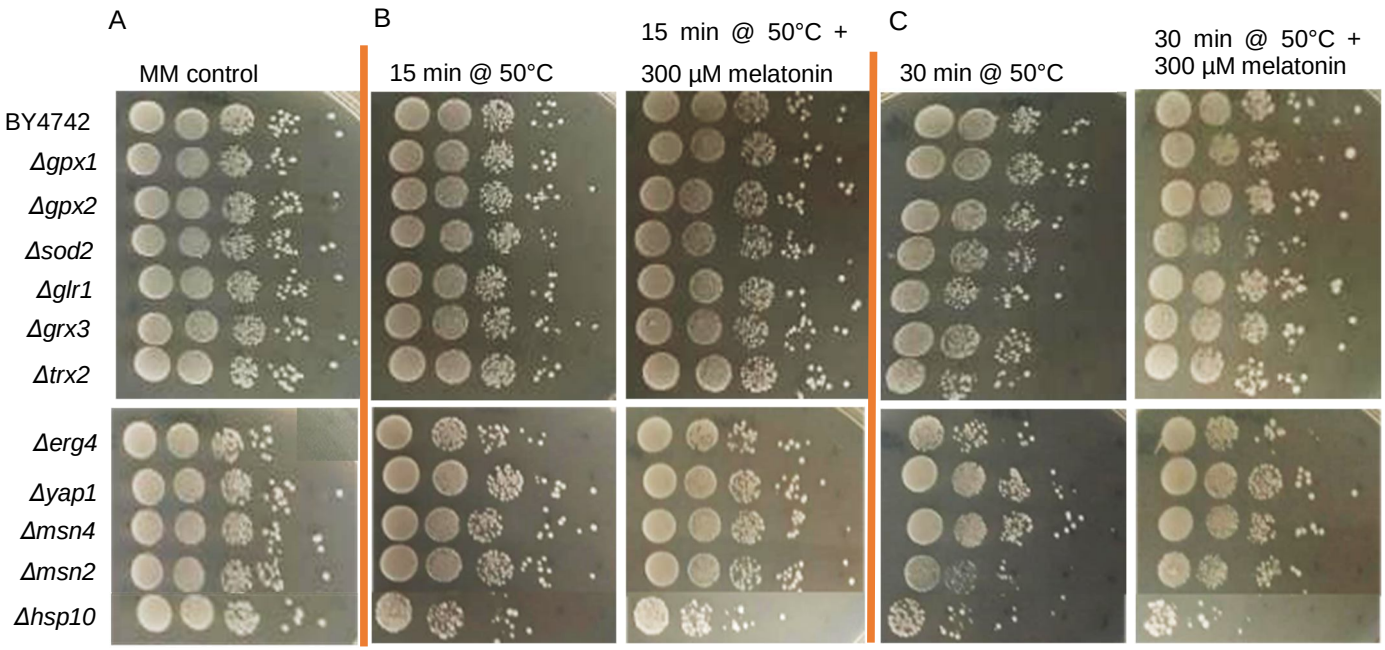

Figure 5.4: The effect of melatonin supplementation on growth of S. cerevisiae laboratory strain BY4742 and single deletion mutants of genes required for the cells defence against oxidative stress and heat shock. MEL treated and untreated cultures were incubated in a 50 °C water bath for 15 and 30 min thereafter ten microliters of five 10-fold serial dilutions were spotted MM agar. A - control cultures grown at 30 °C; B – cultures heat shocked for 15 min at 50 °C; C - cultures heat shocked for 30 min at 50 °C.

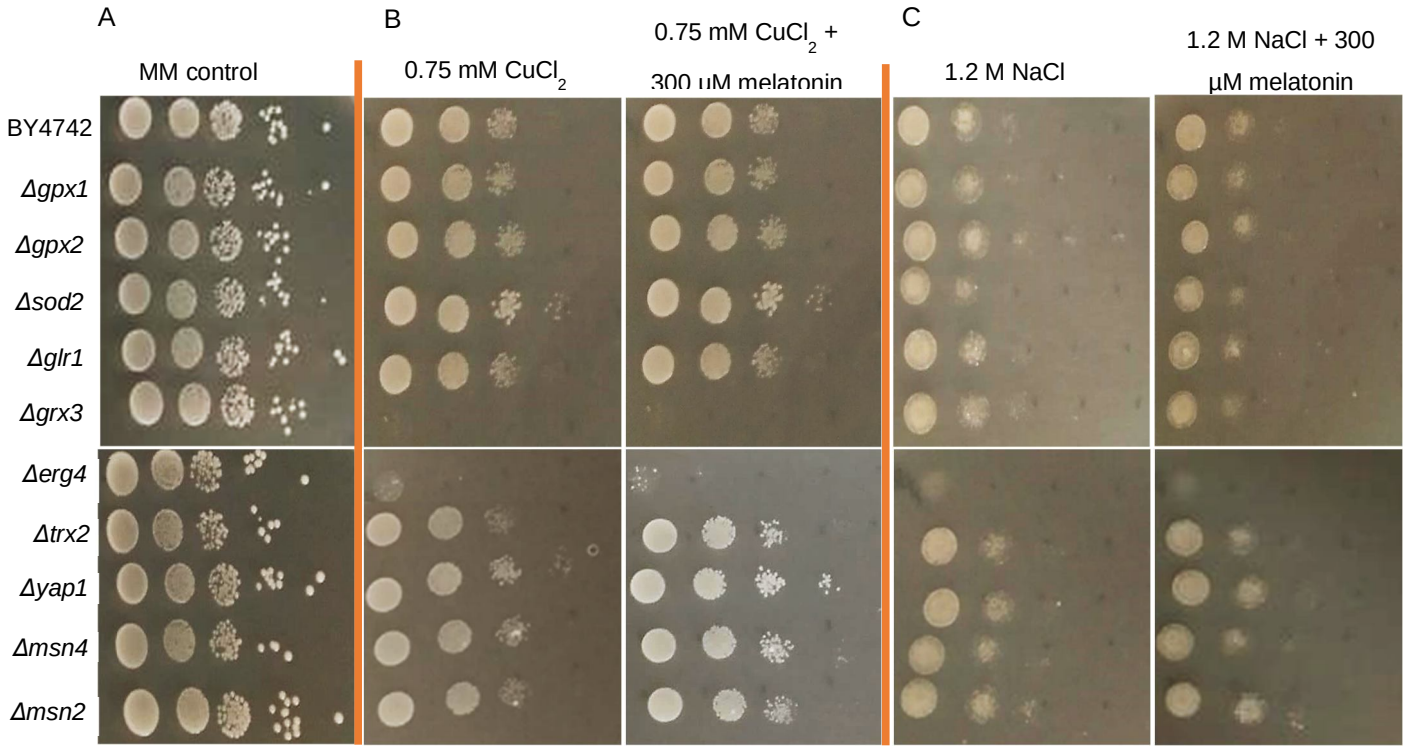

Figure 5.5: The effect of melatonin supplementation on growth of S. cerevisiae laboratory strain BY4742 and single deletion mutants of genes required for the cells defence against oxidative stress. Ten microliters of five 10-fold serial dilutions were spotted MM agar only (A) or MM containing $CuCl_2$ (B) and NaCl (C) with and without 300 µM MEL.

5.4.2 Impact of oxidative and reductive stressors on growth

The transcriptomic analysis (Chapter 4) found that MEL led to the up-regulation of methionine biosynthetic pathway genes before the induction of stress. Once stress was induced, the presence of MEL led to increased expression of glutathione related oxidative stress response genes (*GLR1, GPX2* and *GRX6*), transcription factors *MSN2/4* and influenced several genes in the ergosterol biosynthetic pathway. This study sought to investigate the impact of MEL on expression of antioxidant genes as well as ergosterol and *MET* genes in response to two oxidative stress agents (H_2O_2 and menadione) and reductive stress agent (DTT) on cultures prepared as described in 5.3.3. Several concentrations of menadione and DTT were tested in order to determine appropriate sublethal concentration of the oxidative and redox stressor respectively (Fig 5.6). Concentrations of 0.1, 0.6 and 1 mM were selected for the menadione, H_2O_2 and DTT experiments respectively. The H_2O_2 concentration was selected based on the data generated in the RNA sequencing experiments (Chapter 4.3.4) in order to validate these experiments.

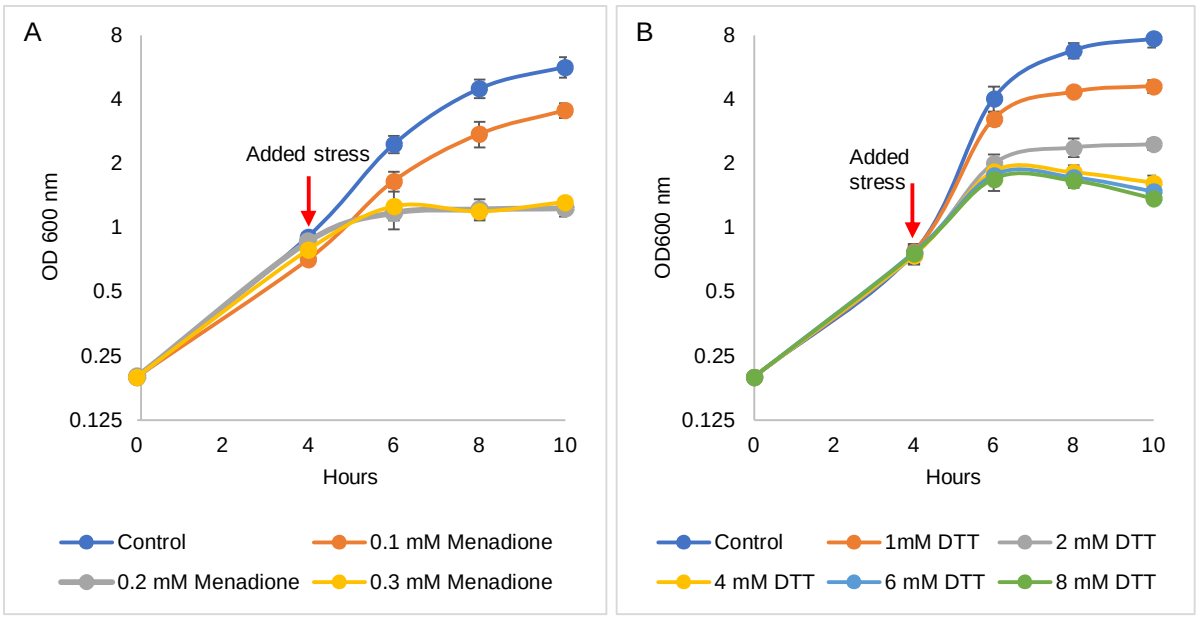

Figure 5.6: Response of exponentially growing *S. cerevisiae* (IWBT Y805) to (A) menadione and (B) dithiothreitol stress in MM.

Stress was induced in MEL treated and untreated cultures and recovery rate was monitored over an 18-h period. MEL treated cultures had a slightly faster recovery rate when cultures were stressed with H_2O_2 and menadione (Fig 5.7A and B). When stressed with DTT, MEL treated cultures reached a higher optical density at in comparison to the untreated cultures (Fig 5.7C).

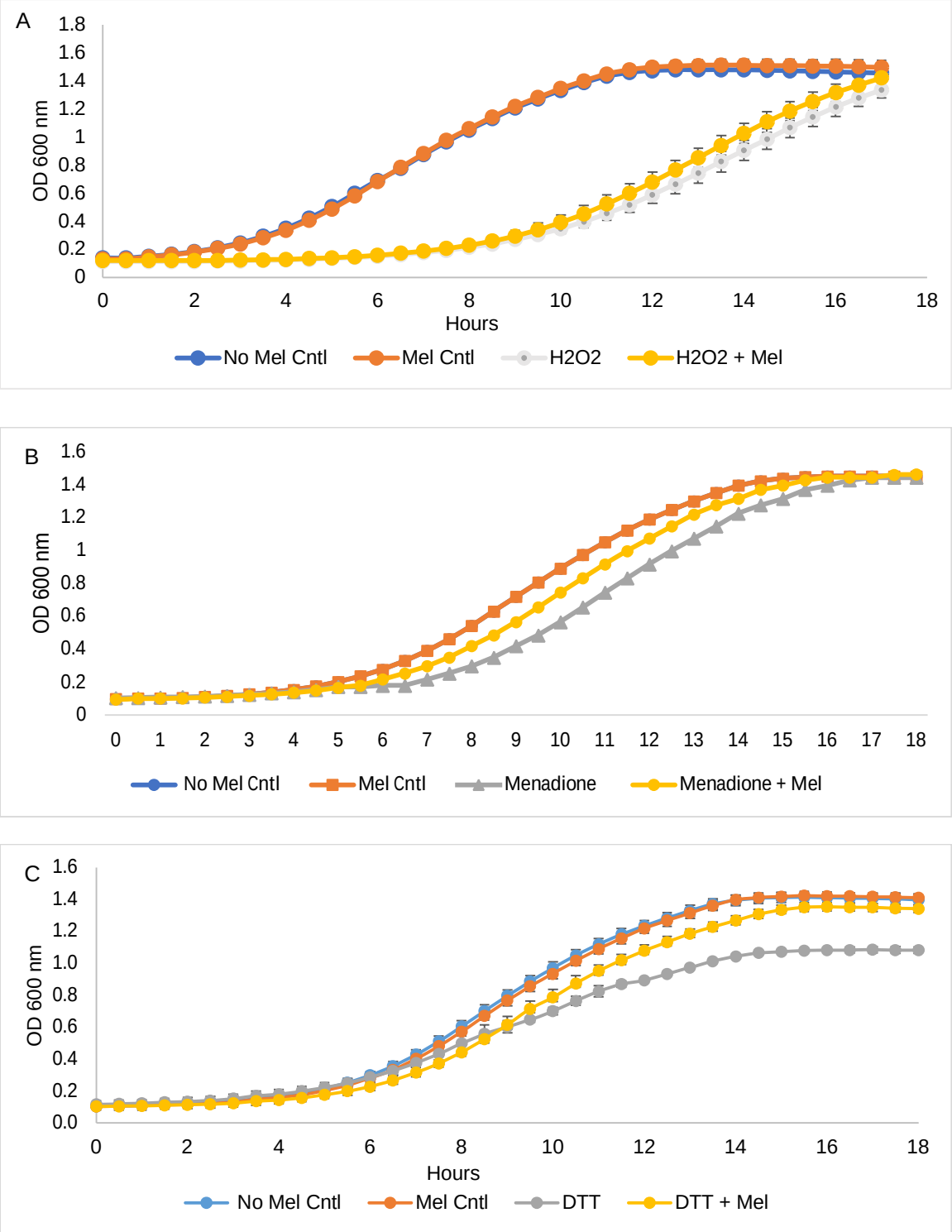

Figure 5.7 Effect of 0.6 mM H_2O_2 (A), 0.1 mM menadione (B) and 1 mM DTT (C) on growth of *S. cerevisiae* (IWBT Y805) cultured in MM under batch conditions in the presence and absence of 300 µM MEL.

Endogenous MEL was not found in the supernatant of any of the samples without MEL supplementation. In the MEL treatment cultures were incubated for 60 min with 300 µM (69.6 mg/L) MEL before the addition of the stressors. At T0 slightly less than half this concentration was still present in the medium suggesting that MEL was taken up or degraded. Upon addition

of stressors, the rate of degradation appeared to be reduced. The break-down product of MEL, 6-hydroxymelatonin, was only quantifiable in the samples supplemented with MEL. The 6-hydroxymelatonin levels quantified at T0 were approximately a half of the levels of MEL quantified at the same timepoint in the H_2O_2 treatment and approximately a quarter of the levels in the menadione and DTT treatments. Levels of 6-hydroxymelatonin increased after the addition of the stressors (Table 5.2).

Table 5.2: Concentrations of MEL and 6-hydroxymelatonin (mean ± SD of triplicate measurements) after addition of H_2O_2, menadione and DTT to *S. cerevisiae* IWBT Y805 batch culture incubated for 60 min in the presence of 300 µM (69.6 mg/L) MEL prior to addition of stressor.

		Melatonin (mg/L)		6-hydroxymelatonin (mg/L)	
Stressor	Time (min)	Melatonin treated	Untreated	Melatonin treated	Untreated
H_2O_2	0	12.79± 1.08	ND[a]	5.93 ± 0.09	ND
	5	11.05 ± 0.95	ND	7.14 ± 0.69	ND
	15	10.95 ± 0.75	ND	7.12 ± 0.15	ND
	30	8.66 ± 0.12	ND	6.76 ± 0.70	ND
Menadione	0	25.71 ± 3.43	ND	5.82 ± 0.03	ND
	5	25.05± 2.83	ND	6.44 ± 0.44	ND
	15	24.14 ± 3.43	ND	6.38± 0.08	ND
	30	21.98 ± 3.27	ND	6.44 ± 0.38	ND
DTT	0	25.76 ± 2.65	ND	6.40 ± 0.09	ND
	5	24.17 ± 2.05	ND	9.58 ± 1.73	ND
	15	24.61 ± 1.64	ND	10.34 ± 0.60	ND
	30	24.22 ± 0.32	ND	8.93 ± 1.48	ND

[a] Below detection limit of assay for accurate quantification

5.4.3 Melatonin up-regulates sulphur assimilation and methionine biosynthesis genes

Quantitative PCR was used for the transcriptional analysis (described in 5.3.3) of genes involved in antioxidant defence such as *SOD2* (mitochondrial superoxide dismutase), *GLR1, GPX1, GPX2, GRX3* and *GRX6.* Expression of genes (*ERG4, ERG 26*) involved in the biosynthesis of ergosterol was also investigated. In the H_2O_2 stressed cultures, the expression levels of *GPX2* and *GRX3* (Fig 5.8 A and E) increased within the 30 min sampling period. MEL treated cultures had significantly higher expression levels of *GPX2* at all timepoints after exposure to H_2O_2 induced oxidative stress. Contrary to was expected, oxidative stress did not result in the immediate induction of the other antioxidant genes (Fig 5.8 B-D, F). Expression

levels of ergosterol genes remained either relatively unchanged with *ERG4* or slightly down-regulated with *ERG26* (Fig 5.8 G and H).

When cultures were exposed to menadione induced oxidative stress, there was an increase in the expression levels of all the genes involved in antioxidant response except for *GRX3* (Fig 5.9). The expression levels of both ergosterol genes were down-regulated after addition of menadione within the studied time period. MEL did not have a significant impact on the expression levels of any of the genes. DTT stress (Fig 5.10) did not induce a strong response in any of the genes except for *GPX2* at 15 min where MEL supplementation significantly decreased expression. MEL supplementation did not seem to have a significant impact on the expression levels of any of the genes after the addiction of stress, but it increased the expression of *GPX2* and *SOD2* before the addition of stress (0 min). Expression of the ergosterol genes decreased slightly in both MEL treated and untreated samples after the addition of stress.

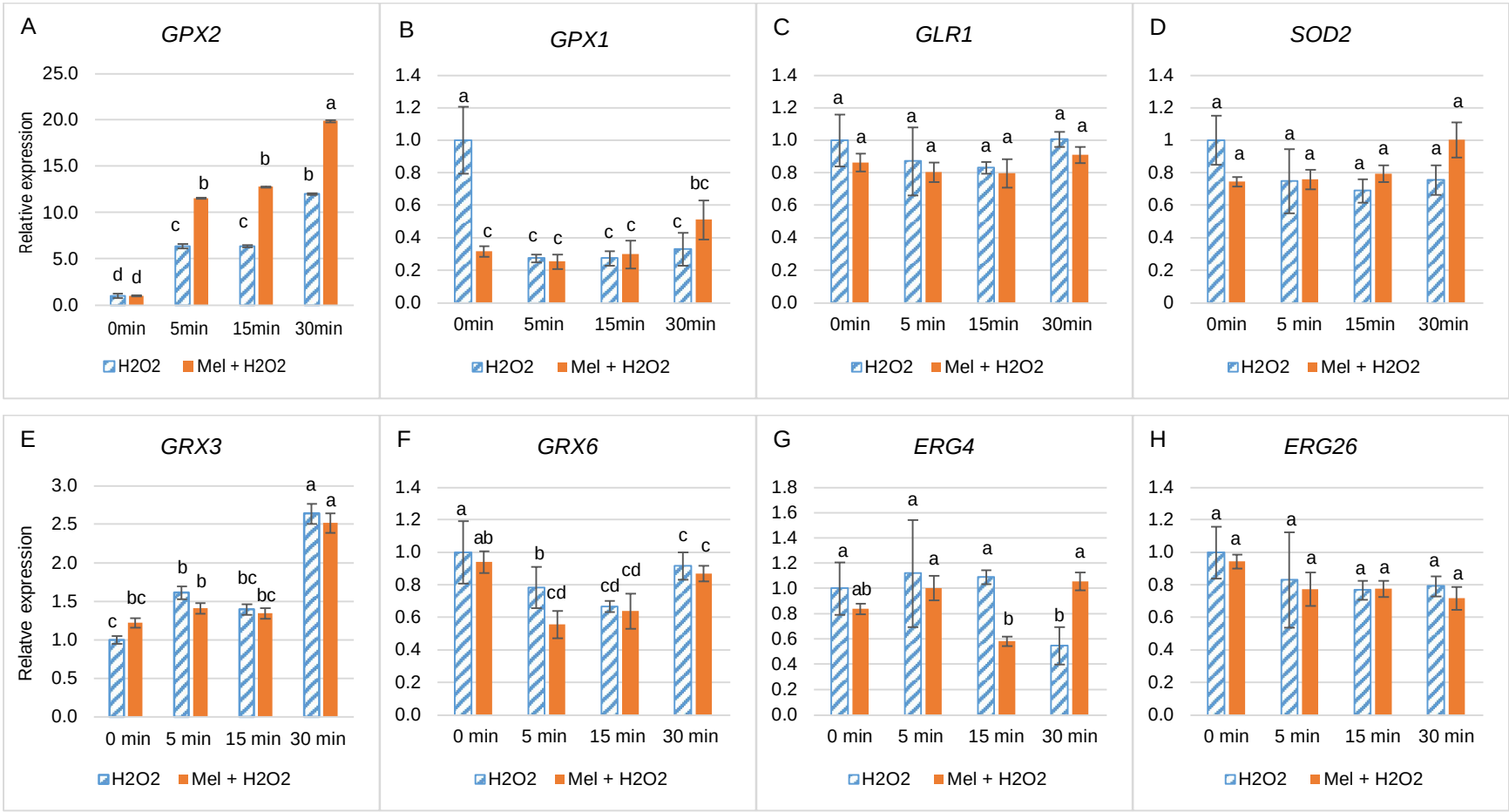

Figure 5.8: Effect of melatonin (MEL) on the expression of genes involved in oxidative stress response and ergosterol biosynthesis before (0 min) and after (5, 15 and 30 min) exposure to 0.6 mM H_2O_2 stress. MEL treated cultures (solid bars) were grown in MM containing 300 µM MEL. Changes are expressed relative to gene expression of the untreated cells before the stress exposure (set as value 1). Different letters indicate significant differences between conditions and timepoints by ANOVA and Tukey's post-test (a–d), $p < 0.05$.

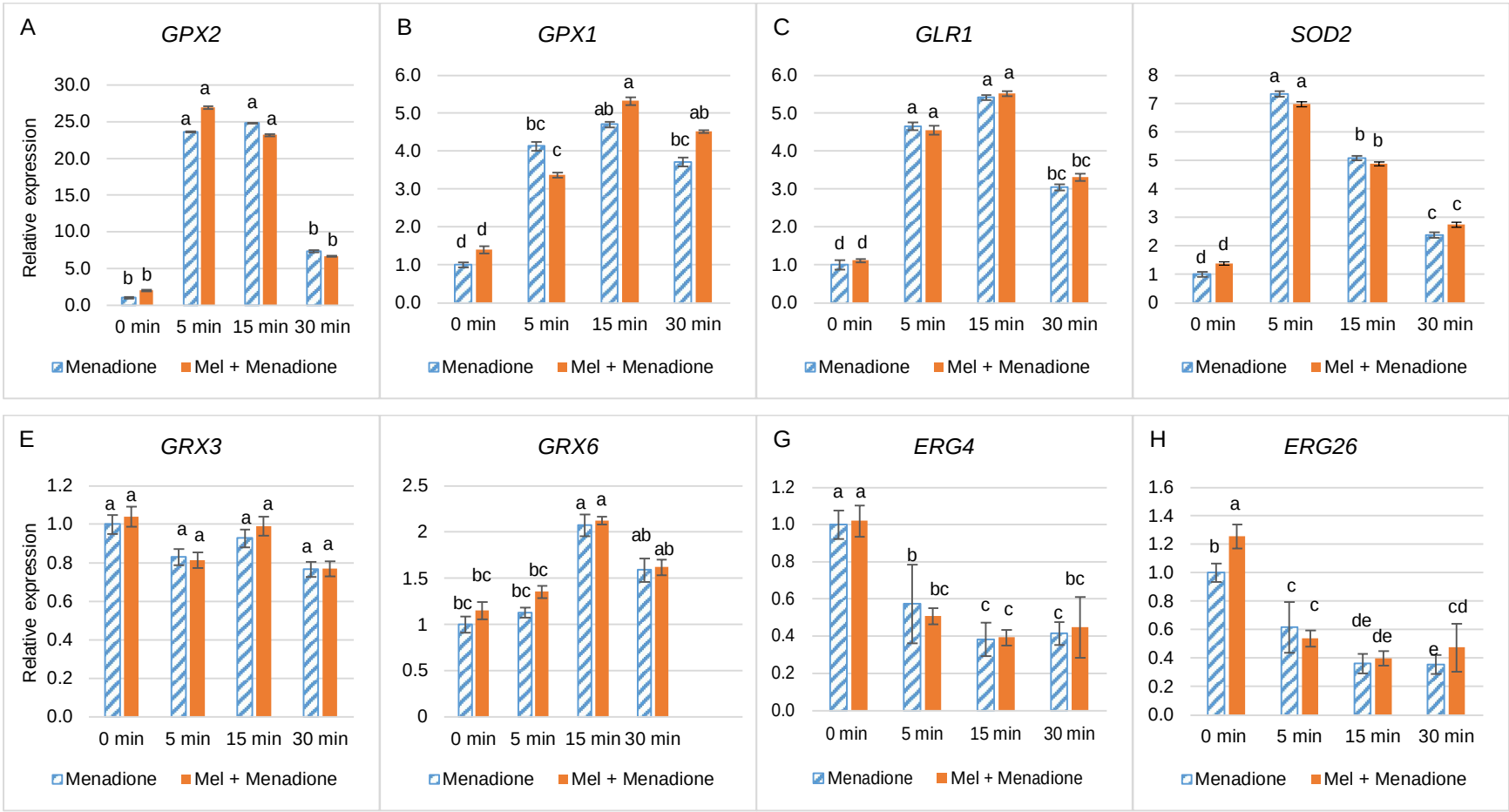

Figure 5.9: Effect of melatonin (MEL) on the expression of genes involved in oxidative stress response and ergosterol biosynthesis before (0 min) and after (5, 15 and 30 min) exposure to 0.1 mM menadione stress. MEL treated cultures (solid bars) were grown in MM containing 300 µM MEL. Changes are expressed relative to gene expression of the untreated cells before the stress exposure (set as value 1). Different letters indicate significant differences between conditions and timepoints by ANOVA and Tukey's post-test (a–e), $p < 0.05$.

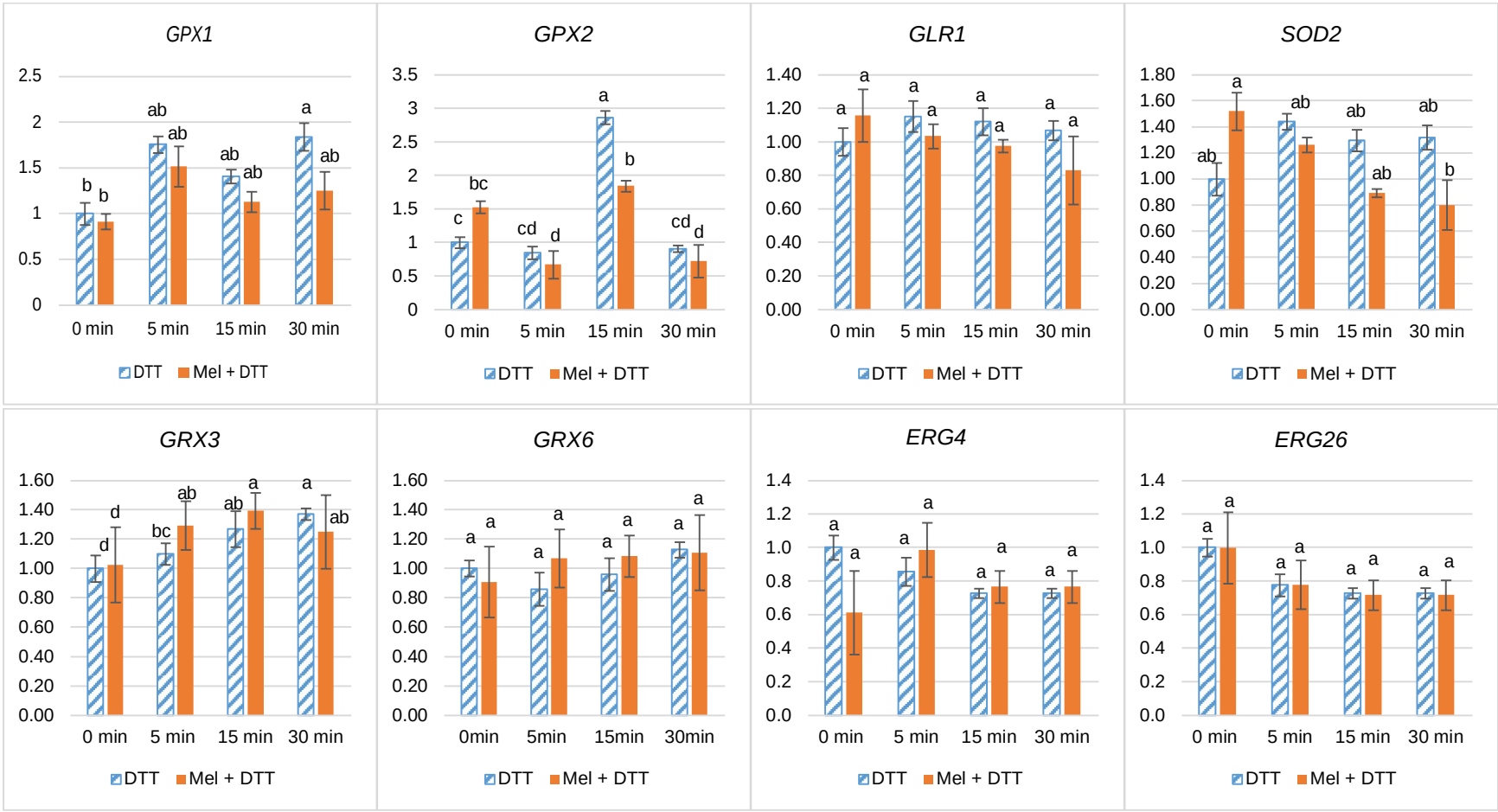

Figure 5.10: Effect of melatonin (MEL) on the expression of genes involved in oxidative stress response and ergosterol biosynthesis before (0 min) and after (5, 15 and 30 min) exposure to 1 mM DTT stress. MEL treated cultures (solid bars) were grown in MM containing 300 µM MEL. Changes are expressed relative to gene expression of the untreated cells before the stress exposure (set as value 1). Different letters indicate significant differences between conditions and timepoints by ANOVA and Tukey's post-test (a–d), $p < 0.05$.

Methionine has been shown to play a central role in stress resistance. It is a precursor for cysteine which is involved in the synthesis of glutathione (Martínez et al., 2017). This amino acid can also act as a scavenger of ROS thus protecting cells from oxidative stress (Campbell et al., 2016). The transcriptomic data in Chapter 4 revealed that MEL enhanced the expression of genes involved in sulphate assimilation pathway in the absence of stress. In this study the expression of *MET2, MET14, MET16, MET17* and *MET28* in MEL treated and untreated cultures, before and after the addition of oxidative stressors (H_2O_2 and menadione) and thiol reducing agent DTT were investigated. MEL enhanced the expression of all the genes in the absence of stress (0 min) in the DTT study (Fig 5.11) and most of the genes except *MET28* in the H_2O_2 study (Fig 5.12) and *MET2* in the menadione study (Fig 5.13) at this timepoint. Even though MEL enhanced the expression of *MET* genes prior to menadione induced stress, this expression level was not significantly above the untreated cultures. But the results demonstrate the effect MEL has on *MET* genes in unstressed cultures. Although expression of all *MET* genes decreased after induction of DTT stress, MEL treated cultures continued to have higher expression of all MET genes in comparison to untreated cultures. In the H_2O_2 experiment, MEL enhanced the expression of *MET14* but did not affect the expression of *MET2*, *MET17* and *MET28* after stress induction. However, the expression of *MET16* was lower in MEL treated cultures. Expression of *MET14*, *MET16* and *MET28* genes was enhanced by MEL treatment after induction of menadione stress but it did not affect the expression of *MET2* and *MET17.*

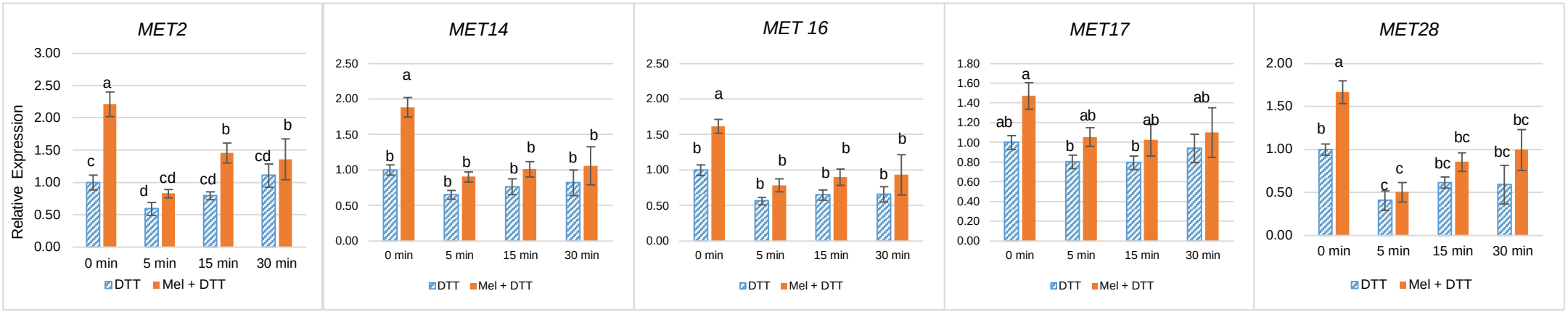

Figure 5.11: Effect of melatonin (Mel) on the expression of genes involved in methionine biosynthesis before (0 min) and after (5, 15 and 30 min) exposure to 1 mM DTT stress. Mel treated cultures (solid bars) were grown in MM containing 300 µM melatonin for 60 min before application of stress. Changes are expressed relative to gene expression of the untreated cells before the stress exposure (set as value 1). Different letters indicate significant differences between conditions and timepoints by ANOVA and Tukey's post-test (a–d), $p < 0.05$.

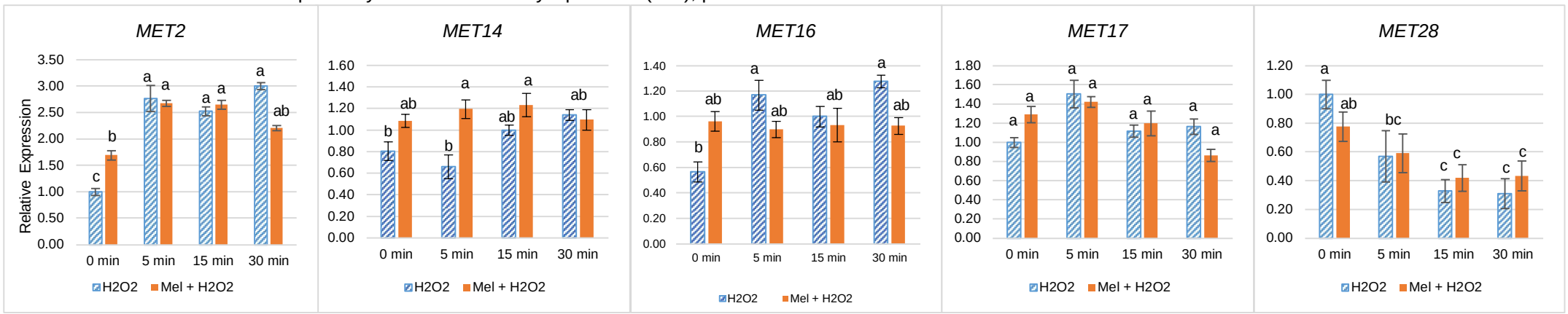

Figure 5.12: Effect of melatonin (Mel) on the expression of genes involved in methionine biosynthesis before (0 min) and after (5, 15 and 30 min) exposure to 0.6 mM H_2O_2 stress. MEL treated cultures (solid bars) were grown in MM containing 300 µM melatonin for 60 min before application of stress. Changes are expressed relative to gene expression of the untreated cells before the stress exposure (set as value 1). Different letters indicate significant differences between conditions and timepoints by ANOVA and Tukey's post-test (a–c), $p < 0.05$.

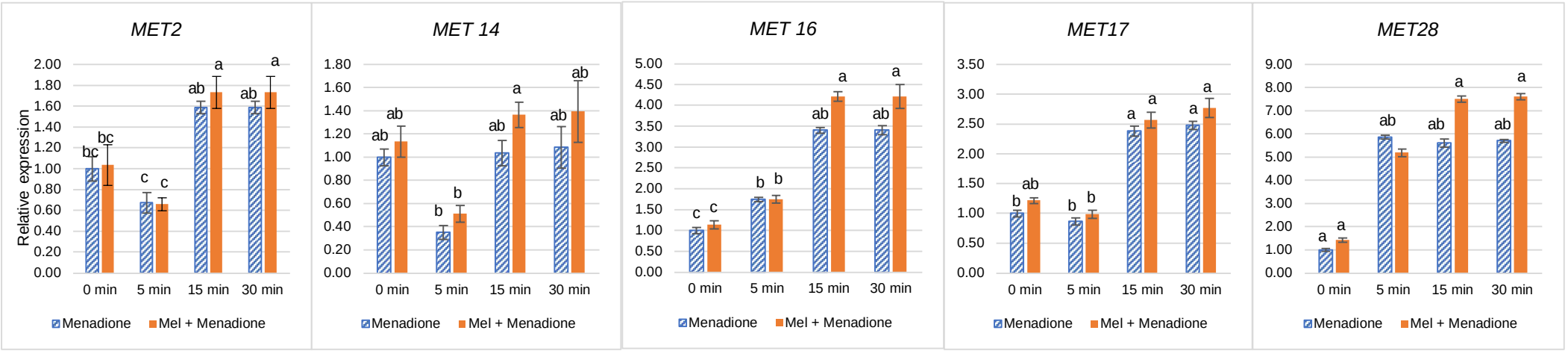

Figure 5.13: Effect of melatonin (Mel) on the expression of genes involved in methionine biosynthesis before (0 min) and after (5, 15 and 30 min) exposure to 0.1 mM menadione stress. Mel treated cultures (solid bars) were grown in MM containing 300 µM melatonin for 60 min before application of stress. Changes are expressed relative to gene expression of the untreated cells before the stress exposure (set as value 1). Different letters indicate significant differences between conditions and timepoints by ANOVA and Tukey's post-test (a–c), $p < 0.05$.

5.5 Discussion

The physiological role of MEL in plants and animals has been studied extensively and its antioxidant capacity is one of the most important biological activities described to date. However, the MEL antioxidant role in the physiology of *S. cerevisiae* has received little attention. Recently, Vazquez et al. (2017) focused on the protective effect of MEL against H_2O_2-induced oxidative stress in *S. cerevisiae* whereas Bisquert et al. (2018) studied the moderation of UV stress and H_2O_2 induced stress by MEL also in *S. cerevisiae*. Here, this study has expanded on the previous authors' work by investigating the impact of other stress agents in *S. cerevisiae* wild type strains, deletion mutants and *Kazachstania* strains in the presence and absence of MEL.

During fermentation, yeast cells are exposed to severe stress conditions such as osmotic pressure, starvation, high acidity and high alcohol concentration. Exposure to some of these stressors generates ROS, which adversely affects the viability and fermentative performance of the cells if their ability to eliminate these reactive species is exceeded. In the plate assays, MEL improved the growth of *S. cerevisiae* (Fig 5.1), *K. aerobia* and *K. servazii* (Fig 5.2) strains in conditions of oxidative and reductive stress. In the case of *Kazachstania* species, the protective effect of MEL against H_2O_2 stress was concentration dependent and high concentrations of this indoleamine seemed to have pro-oxidant activity. This concentration dependent pro-oxidant activity of MEL was reported in *in vitro* studies on human cells (Osseni et al., 2000; Zhang and Zhang, 2014) but recently, Vázquez et al. (2017) found that low doses of MEL altered basal glutathione levels and slightly increased ROS levels. However, these low non-toxic ROS levels may act as signalling molecules (Collinson and Dawes, 1992; Flattery-O'Brien et al., 1993; Davies et al., 1995; Jamieson, 1992, 1998) allowing the cells to adapt and build resistance to subsequent lethal exposure to ROS. The appropriate concentration for optimal pro-oxidant activity in yeast and the mechanisms involved require further investigation. In plants, MEL has been shown to protect against saline stress (Chen et al., 2018; Zhan et al., 2019); however in this study, no effect was observed in the case of NaCl or acetic acid stress which suggests a that MEL does not confer protection against all stressors in yeast.

In response to increases in ROS levels, cells activate enzymatic and non-enzymatic defence mechanisms in order to maintain a proper redox balance. Enzymatic defence systems consist of enzymes that remove oxygen radicals and repair the damage caused whereas non-enzymatic systems including small, soluble molecules act as radical scavengers and remove the ROS from the solution (Jamieson, 1998). In Chapter 4, MEL was found to influence the expression of transcription factors *MSN2/4*, glutathione and glutaredoxin enzymes. This

current study focused on the impact of MEL on antioxidant genes in two ways. Firstly, by using *S. cerevisiae* strains harbouring defects in the antioxidant machinery which can emulate the altered intracellular redox environments, the capacity of MEL could be assessed to protect these cells against oxidative insults. MEL improved survival of wild type and antioxidant deletion mutants to H_2O_2, menadione, DTT and thermal stress especially the glutathione related genes (Fig 5.3 and 5.4). Although studies have shown that MEL helps maintain GSH redox balance in yeast during oxidative stress (Vázquez et al., 2017), this response is common among antioxidants. In this study, the resistance towards stress compounds of deletion mutants of enzymes directly involved in the recycling of oxidized GSSG back to reduced GSH was increased by MEL supplementation. This impact on GSH/GSSG balance was observed in response to other antioxidants. Vitamin C and E supplementation to GSH-depleted, H_2O_2-stressed human lens cells restored GSH:GSSG ratios to pre-stress levels and increased the resistance of these cells to H_2O_2 (Shang et al., 2003). Quercetin (an antioxidant found fruits, vegetables and grains) treatment stimulated the stress responsive machinery in *S.cerevisiae* by increasing the levels of GSH and GSSG cultures prior to H_2O_2 stress which may have increased resistance and lifespan of the cultures (Belinha et al., 2007; Amari et al., 2008). It therefore appears that there are no enzyme activities that have a specific requirement for MEL in yeast. Although several animal and plant studies have shown that MEL can protect cell structures from copper induced toxicity (Walters-Laporte et al., 1998; Mayo et al., 2003; Zatta et al., 2003; Romero et al., 2014) and salt stress (Zhang et al., 2015; Chen et al., 2018), similar protective activities against copper stress were not observed in this study which could be as a result of experimental methodology used. In liquid medium, exposure of cultures to stress compounds as well as exogenous antioxidants is distributed uniformly in the medium whereas on solid plates this exposure could be buffered by the compact nature of colony growth on plates.

The protective effects of MEL may occur via several mechanisms such as directly scavenging ROS (Reiter et al., 2016), stimulating the synthesis of other antioxidants such as glutathione or increasing the transcription level of genes related to the antioxidant response (Rodriguez et al., 2004; Vázquez et al., 2017). The transcriptomic study in Chapter 4 suggested that MEL supplementation up-regulated genes involved in sulphur metabolism (*MET1, MET2, MET14, MET16, MET17* and *MET28*) in unstressed cells. The *MET* genes highlighted in this study are involved in the general sulphate assimilation pathways in yeast and are involved in the biosynthesis of pre-cursor compounds used to synthesise sulphur containing amino acids such methionine and cysteine. *MET14* and *MET16* are involved in the conversion of sulphate to sulphide whereas *MET2* converts L-homoserine to O-acetyl-L-homoserine and *MET17* converts this metabolite to L-homocysteine. *MET28* is a bZIP

transcriptional activator which participates in the regulation of sulphur metabolism. L-homocysteine can be methylated to methionine via cobalamin-dependent or -independent enzymes or condensed to form cysteine (Thomas and Surdin-Kerjan, 1997; Ono et al., 1999; Rückert et al., 2003). Cysteine is a component of antioxidant molecules such as glutathione, glutaredoxin, thioredoxin and Yap1p (Sha et al., 2013). Methionine has also been shown to chelate lead from tissues thereby decreasing oxidative stress (Patra et al., 2001) as well as increase the resistance of *S. cerevisiae* to the thiol reducing agent diamide (Campbell et al., 2016). This study has confirmed the impact of MEL on the expression of these genes in unstressed cultures (Fig 5.11, 5.12 and 5.13; 0 min). After the induction of stress by H_2O_2, menadione and DTT, this effect was maintained only in the *MET14* gene. When assessing the transcriptional response of various antioxidant genes MEL supplementation only enhanced the expression of *GPX2* prior to induction of menadione and DTT stress (Fig 5.9 and 5.10). After the stress induction, higher transcript levels were obtained in MEL treated culture at the 5 min timepoint in H_2O_2 (Fig 5.8) and menadione stress and 15 min and 30 min timepoints in the H_2O_2 stress. The data generated thus far shows that the impact of MEL on the induction of antioxidant enzymes is similar to the response elicited by other antioxidants such as Vitamin A, C and E as well as caffeic acid. Such studies found that Vitamin A and E induced the activity of superoxide dismutase, catalase and glutathione peroxidases in *S. cerevisiae* leading to improved survival after H_2O_2 stress (Bronzetti et al., 2001), and vitamin C and caffeic acid were found to improve the survival of *S. cerevisiae* strains deficient in superoxide dismutase (Δ*sod1*), catalase A (Δ*cta1*), and double-deficient in Old Yellow enzyme 2 and glutathione reductase 1 (Δ*oye2 glr1*) against H_2O_2 stress (Amari et al., 2008).

In conclusion, it appears that the antioxidant activity of MEL and its impact on gene expression is a generic feature of ROS-scavenging antioxidant molecules, and not a specific cellular response to MEL. In fact, apart from the impact on *MET* genes, none of the responses described in this chapter suggests any specific role for MEL in yeast physiology. MEL may protect yeast cells from various ROS inducing compounds. However, this study does describe for the first time the influence of MEL on the sulphate assimilation pathway which plays a critical role in the synthesis of sulphur containing antioxidants. The induction of *MET* and some of the antioxidant genes (*GPX1, GPX2, GRX3* and *SOD2*) before addition of stress could have primed the cells to tolerate subsequent stress more efficiently. After the addition of stress compounds, the free radical scavenging activities of MEL and 6-hydroxymelatonin in addition to induction of antioxidant genes improved the recovery rate of *S. cerevisiae* from H_2O_2, menadione and DTT stress. However, the findings also raise intriguing questions about the impact of MEL on sulphur metabolism in yeast which needs further investigation.

5.6 References

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Chapter 6

General discussions and conclusions

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6.1 General discussion and future works

Despite the increase in the number of research groups investigating melatonin production by yeast (Sprenger et al., 1999; Rodriguez-Naranjo et al., 2011; Bisquert et al., 2018; Vázquez et al., 2017, 2018; Fernandez-Cruz et al., 2019; Muñiz-Calvo et al., 2019), the biosynthetic pathway remains unclear and its physiological role has not been defined. The goal of this study was to characterise growth conditions that stimulate melatonin (MEL) production by yeast, identify its biosynthesis pathway and define its physiological role.

Biotic and abiotic factors have been shown to consistently induce MEL production in animals and plants. However, none of these factors or the pulsing of precursor molecules in media could establish consistent production by yeast in this study. Bisquert et al. (2018) stated that they were also unable to induce reproducible MEL synthesis in *S. cerevisiae,* but they suggested that pulses of MEL pathway intermediates as well as tryptophan induced MEL production. In our study, when yeast cultures were cultivated in laboratory media with different concentrations of tryptophan, a direct correlation between concentrations of MEL produced and tryptophan levels in the medium was not established. In some cases, higher levels of MEL were quantified in the medium with low levels of tryptophan and vice versa. Other authors investigating MEL production by *S. cerevisiae* Lalvin QA23 (Lallemand Inc) found variations in the production patterns and concentrations (0-700 ng/mL) during fermentation (Fernández-Cruz et al., 2017, 2018; Muñiz-Calvo et al., 2019; Valera et al., 2019). This same yeast strain and other *S. cerevisiae* strains also showed inconsistent production patterns in our experimental conditions. Other studies on the production patterns suggested a link with yeast growth phase (Rodriguez-Naranjo et al., 2012; Vigentini et al., 2015), a finding that is not supported by my data. In the instances where MEL was detected in our batch culture experiments, the highest concentrations were not growth phase related. In some experiments in minimal medium, an inverse relationship between intracellular and extracellular concentrations of MEL was observed. In these experiments intracellular concentrations were initially high at the early timepoints but later declined in parallel with increases extracellular concentrations. The data suggest that intracellular MEL, when and if produced, is released at a later stage. However, no MEL transport mechanism in yeast has been described and the increase in extracellular concentrations of MEL could be a result of active transport, passive diffusion or cell lysis. To get a better understanding of whether cell death and lysis is involved,

fluorescence microscopy could be used in further experiments to determine the number of live and dead cells at each timepoint. If MEL is detected, an analysis of the intracellular and extracellular levels over time in comparison to the number live and dead cells could shed more light on this topic.

Cultivation of yeast in continuous culture conditions allowed us to test three parameters (circadian rhythm, tryptophan concentration and oxidative stress) that have been associated with MEL synthesis in various organisms. Again, and although continuous cultivation provides in principle the best controlled of all culture conditions, the data did not reveal any reproducible production. Circadian rhythm is one form of an oscillatory production pattern, and several metabolites have been described whose production or free concentration follows oscillations of various time intervals, from seconds to diurnal (Murray et al., 2003; Dick et al., 2013). One explanation for the non-reproducibility of our data may therefore be a hidden oscillatory rhythm of MEL release or production. This data allowed us to exclude circadian and, probably, most hour-long rhythms, but do not exclude the possibility of more short-term oscillations of MEL. Arguably, they nevertheless appear to exclude the possibility that such oscillations are triggered by the investigated stressors. All biological repeats were always taken at the same time points, and a specific trigger of oscillation should therefore have yielded approximately similar values, whether “on” or “off”, at least between biological repeats. Other forms of oscillations that occur in yeast include glycolytic and respiratory oscillations and the frequency of these oscillation could be less than a 1 min long. The frequency of these oscillations could be affected by parameters such as rate of glucose addition, temperature, pH or protein concentration (Murray et al., 2003; Richard, 2003). Although all care was taken to ensure that environmental parameters were the same in our experiments, minute fluctuations in these parameters could have caused chaotic oscillations in MEL production. Observations of macroscopic oscillations were also found to be cell density dependent. Aon et al (1992) found that NADPH oscillated in single cells even after the macroscopic oscillations had died out. Oscillations of MEL could be occurring in a similar manner at a single cell level but because of slight differences in frequency and the absence of a synchronisation mechanism, they are not apparent at a macroscopic level. The possibility of more short-term oscillations of MEL may deserve further investigation but could not be included in this study due to practical and logistical limitations. To verify if yeast produce MEL in oscillatory patterns, experiments with frequent sampling points would need to be performed, or a visual reporter system would need to be developed. Sampling strategies in these experiments should capture rapid (second or minute) and slow (hourly) changes in MEL levels in order to describe possible oscillatory patterns.

Bioinformatic analysis of enzymes involved in the biosynthetic pathway in plants and animals was unsuccessful in identifying functional orthologs in yeast. The likely absence of a dedicated pathway in yeast suggests that MEL could be a by-product of unrelated metabolic activities (Ganguly et al. 2001), and may also explain inconsistent production patterns. Data generated in our study suggest that the pathway in yeast is likely similar to the pathway from tryptophan to serotonin via 5-hydroxytryptophan, but other studies have found that serotonin could also be synthesised from tryptamine (Muñiz-Calvo et al., 2019). When characterising scAANAT activity, Ganguly et al. (2001) found that this enzyme had a low substrate affinity for serotonin and tryptamine and suggested that MEL synthesis is caused by the opportunistic action of otherwise unrelated metabolic enzymes with broad functions e.g. aromatic hydroxylation, decarboxylation, N-acetylation or O-methylation. Tryptophan hydroxylase belongs to the aromatic amino acid hydroxylases family of enzymes and require tetrahydropterin cofactors to function which is absent in yeast. This enzyme converts tryptophan to 5-hydroxytryptophan. The apparent absence of tryptophan hydroxylases in yeast suggest that other hydroxylases maybe involved in this process. *S. cerevisiae* has several decarboxylases, but none have been shown to have tryptophan decarboxylase activity. In mammals, this enzyme catalyses the decarboxylation of various aromatic amino acids (Berry et al., 1996) whereas plants have various isoforms of this enzyme which seem to differ in substrate specificity (Hardeland, 2016). The broad specificity of these enzymes makes it difficult to narrow down potential targets in yeast and characterise their substrate specificity. However, when a potential target is identified, the protein of this enzyme could be purified and further characterised for its substrate affinity. These characterisation experiments would determine if the *S. cerevisiae* enzymes have the substrate affinity and enzyme kinetic features to catalyse melatonin pathway reactions.

Physiological stress experiments could not consistently induce MEL synthesis by yeast, but the application of exogenous MEL to unstressed cultures revealed that MEL may increase the expression of genes in the sulphate assimilation pathway which leads to the production of methionine and cysteine. These amino acids are involved in production of glutathione and related antioxidants. Previously MEL was shown to alleviate H_2O_2 stress by stimulating sulphur containing antioxidants genes such as *GSH1, GPX1, GPX2, GLR1 and TRX2* (Vázquez et al., 2017; Bisquert et al., 2018). Although this link was not established in these studies, it indicates a potential role of MEL in sulphur assimilation in yeast. Several studies have described the effect of environmental stressors such as heavy metal exposure, oxidative stress and sulphur or nitrogen deficiency on sulphate assimilation pathways (Mendoza-Cózatl et al., 2005). Recently Hasan et al. (2018) found that MEL supplementation stimulated the uptake and assimilation of sulphur in tomato plants by regulating the expression

of genes encoding enzymes involved in sulphur transport and metabolism, enhancing the plants tolerance to low-sulphur-induced stress. By inducing the sulphate assimilation genes, MEL may prime the cells antioxidant machinery to respond to subsequent stress more efficiently resulting in improved survival of yeast cultures. To determine the impact of MEL on sulphur uptake, intracellular and extracellular concentrations of sulphur containing metabolites such as cysteine, methionine and glutathione should be analysed before and after addition of MEL. By testing various concentrations of MEL (physiological and non-physiological), we would determine if the impact observed correlates with concentrations of MEL used. Once the impact of MEL supplementation on sulphur containing metabolites in non-stressed cultures is determined, further analysis on this response under various stress conditions should be conducted.

It should also be kept in mind that in our experiment the concentration of exogenous MEL was significantly higher than any reported physiological MEL concentration, whether reported as derived from yeast biosynthesis or environmental. The data are therefore somewhat physiologically questionable and may simply reflect the action of any generic antioxidant molecule. By functioning as a direct radical scavenger MEL may have changed the redox state of cells which may have changed the specific activity of antioxidant genes. Earlier studies on transcriptomic response of yeast to oxidative stress and the impact of antioxidants were performed in batch culture and used microarray to measure the response (Franken, 2009; Taymaz-Nikerel et al., 2016; Vilaça et al., 2012) and the concentrations of antioxidants used in these studies varied widely. These studies showed that antioxidants enhanced the expression of antioxidant genes and induced trehalose biosynthesis in response to oxidative stress, which is consistent with activities of generic antioxidants and similar responses were observed in my study. By performing experiments in continuous culture conditions which provides a constant environmental framework, we were able to monitor temporal transcriptional response to oxidative stress and cellular recovery as well as the impact of MEL on these processes. Analysis of the temporal response of *S. cerevisiae* in our conditions revealed that MEL may enrich steroid biosynthetic processes and catabolic processes soon after the addition of stress. In the later response phase, metabolic processes were enriched resulting in faster recovery of MEL treated cells in comparison to the untreated cultures. Although the concentrations of exogenous MEL used in this study where higher than physiological concentrations produced by yeast, the concentration of MEL quantified in the supernatant before stress was elicited was significantly lower (1.7 µM (Table 4.3)) than 300 µM which may be caused by cellular uptake or metabolism of MEL. This concentration is much closer to the endogenous concentrations reported in this study as well as other studies (800 µg/L or 3.44 µM MEL) (Valera et al., 2019). Intracellular levels of MEL were not determined in

our physiological experiments but further experiments on the impact of exogenous MEL on yeast physiology should determine both intracellular and extracellular levels. This will determine how much MEL is taken up by the cells and how much is metabolised to other metabolites.

MEL is a non-essential metabolite, and this study used a multipronged approach to understand its physiological role. Conditions that stimulate consistent melatonin production in yeast remain elusive. However, our data has shed some light on the molecular mechanisms behind its antioxidant activity. The data suggests that MEL does not act through any specific stress responsive pathway and could not be linked any specific genetic interaction or regulation. However, the study described for the first time the impact of MEL on sulphate assimilation pathways. The concentrations of MEL used in this study are above the physiological levels reported in yeast, but we observed a significant improvement in the recovery rate of stressed yeast cells in comparison to untreated cells. This allowed us to investigate the underlying molecular mechanisms behind this improved survival. It is important that further studies are done in determine if physiological concentrations of MEL will have similar impacts on sulphate assimilation and the expression of antioxidant genes.

The initial idea of this study had been to identify consistent production patterns of MEL in *S. cerevisiae*, and/or of “screenable” phenotypic responses of *S. cerevisiae* to MEL addition. This would have been followed by the implementation of traditional genetic approaches to identify genes involved in the biosynthesis pathway or in MEL-induced physiological adaptations using the unmatched power of yeast genetics, including mutant selection and identification of genes. In the absence of such conditions and phenotypes, such traditional genetic approaches will remain impossible to implement. Further studies could however further characterise cellular responses to various concentrations (physiological and non-physiological) of exogenous MEL, and its uptake and subsequent release into different media under different environmental conditions. This might lead to a better understanding on the physiological role and identify possible oscillatory patterns of MEL production in yeast.

In the absence of additional data, and considering the full amount of information and data generated in this study and published thus far, our conclusion however is that there are no strong indications that MEL plays any specific metabolic or physiological role in yeast, and that its production is not a controlled response to environmental parameters, but rather a by-product of unrelated metabolic activities. Its physiological impacts indeed appear to be limited to a generic antioxidant response common to ROS scavenging molecules.

6.2 References

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